Sperm-egg interaction in birds: assays and mechanisms

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I certify that this thesis is a true and accurate version of the thesis approved by the examiners

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ABBREVIATIONS, SYMBOLS AND NOTATION

AI	Artificial insemination
AR	Acrosome reaction
BCA	Bicinchoninic acid
BES	N,N-Bis(2 hydroxyethyl)-2 aminoethane sulphonic acid
BSA	Bovine serum albumin
Con A	Concanavalin A (*Canavalia ensiformis*)
CTC	Chlortetracycline
DIC	Differential interference contrast
DMEM	Dulbecco's Modified Eagles' Minimal Essential Medium, buffered with 10 mmol l^{-1} HEPES
FITC	Fluorescein isothiocyanate
Fuc	Fucose
Gal	Galactose
Galtase	β-1,4-Galactosyltransferase
GD	Germinal disc
Glu	Glucose
GlcNAc	N-Acetylglucosamine
IAM	Inner acrosomal membrane
INT	2-[*p*-iodophenyl]-3-[*p*-nitrophenyl]-5-phenyltetrazolium chloride
IPVL	Inner perivitelline layer
IVF	*In vitro* fertilization

IVM	*In vitro* maturation
LH	Luteinising hormone
LPA	*Limulus polyphemus* agglutinin
LTA	*Tetragonolobus purpureas* agglutinin
Man	Mannose
NaCl-TES	0.15 mol l^{-1} NaCl with 20 mmol l^{-1}TES (N-Tris[hydroxymethyl]-methyl-2-aminoethanesulfonic acid); pH 7.4
NeuNAc	N-acetylneuramic acid
OAM	Outer acrosomal membrane
OPVL	Outer perivitelline layer
PBS	10 mM Phosphate buffered saline, pH 7.4
PM	Plasma membrane
PMS	Phenazine methosulphate
PMSF	Phenylmethylsulfonyl fluoride
PNA	Peanut agglutinin (*Arachis hypogaea*)
PNGase F	Peptide - N- Glycosidase F
PSA	*Pisum sativum* agglutinin
PVL	perivitelline layer
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SSTs	Sperm storage tubules
STA	*Solanum tuberosum* agglutinin
S-WGA	Succinyl-*Triticum vulgaris*

TEM	Transmission electron microscopy
UEA II	*Ulex europaeus* II
VMOI	Vitelline membrane outer protein I
WGA	Wheat germ agglutinin (*Triticum vulgaris*)
WPVL	Whole perivitelline layer
ZP	Zona pellucida

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ABSTRACT

Considerable progress has been made in mammalian species since the advent of *in vitro* sperm-egg interaction systems. Although *in vitro* systems for studying sperm-egg interaction in chickens have been developed, they have not been characterised and are limited by the availability of material as only one fertilised egg and a few follicles can be obtained per bird and the bird has to be sacrificed in order to obtain them. Therefore, inner perivitelline layer (IPVL) separated from laid chicken eggs was investigated as a readily-available material for studying spermatozoa-egg interaction *in vitro*. This layer was found to have a similar response to hydrolysis by spermatozoa as IPVL from ovulated and follicular ova in terms of the number of points of hydrolysis produced in a 5 min assay at 40°C. Initiation of hydrolysis of the layer was found to have occurred within 2.5 min, with only the size of the holes, not the frequency, increasing with increased incubation times. The number of points of hydrolysis was found to be positively correlated with the concentration of spermatozoa in the assay. IPVL taken from the same laid egg was found to have a similar response to hydrolysis by spermatozoa, but greater variability was found when fragments of IPVL were taken from different laid eggs. Up to twenty replicate pieces of IPVL can be obtained from a single laid egg, which could be used as an 'experimental unit', providing a plentiful source of material for studying sperm-IPVL interaction *in vitro*.

The *in vitro* sperm-IPVL assay, utilising IPVL from laid eggs, was found to be a suitable assay for assessing sperm quality, and therefore potentially,

the fertilizing ability of spermatozoa. In fresh ejaculates of semen, individual males were found to have a different and characteristic ability to hydrolyse the layer and the results were linearly correlated with standard sperm quality assays. The number of holes formed in the IPVL *in vitro* also correlated linearly with the number of holes formed in the IPVL of eggs fertilized *in vivo*, in inseminated hens; and logarithmically with the percentage of fertile eggs laid. The sperm-IPVL assay was also able to detect more damaged spermatozoa in chicken and turkey semen, which had been stored at 5°C, under both aerobic and anaerobic conditions, than did other tests of sperm quality, which are known to overestimate the fertilizing ability of stored avian semen. The sperm-IPVL assay is therefore likely to be highly discriminatory against compromised spermatozoa and the fact that the test is 'low-tech', inexpensive and simple to perform should ensure its ready application to avian fertility systems.

Chicken spermatozoa were able to hydrolyse the IPVL separated from laid eggs of several avian species. The heterologous gamete combinations showed order dependent, but not species dependent specificity at the level of sperm-IPVL interaction. Mammalian spermatozoa were unable to hydrolyse the IPVL from laid chicken eggs.

Co-incubation of freshly ovulated ova and chicken spermatozoa resulted in preferential hydrolysis of the IPVL overlying the germinal disc (GD) region of the ovum. However, isolated fragments from the same ova incubated with spermatozoa under the same conditions resulted in an

increase in IPVL-holes to numbers comparable to the GD region. Furthermore, when fragments of IPVL with the yolk still attached were incubated with spermatozoa, hydrolysis of the layer was severely inhibited. This suggests that rather than being attracted to the IPVL overlying the GD region of the ovum, spermatozoa are inhibited from hydrolysing the IPVL at areas away from this region by the presence of yolk material.

Fluoroscein isothiocyanate-conjugated peanut agglutinin (FITC-PNA) was able to reliably detect acrosome-reacted chicken spermatozoa. PNA was found to bind to the exposed acrosomes of acrosome-reacted, but not acrosome-intact spermatozoa. At 40°C chicken spermatozoa co-incubated with IPVL required extracellular Ca^{2+} for induction of the acrosome reaction (AR). However, at 30°C, the IPVL was the sole requirement for acrosomal exocytosis. FITC-PNA was also able to detect acrosome-reacted turkey and quail spermatozoa.

Carbohydrates have been shown to play a crucial role in sperm-IPVL interaction in chickens. Removal of N-linked glycans from the IPVL severely inhibited the ability of spermatozoa to hydrolyse the layer. Wheat germ agglutinin (WGA) completely inhibited hydrolysis of the IPVL by spermatozoa, and the addition of D-N-Acetylglucosamine (D-GlcNAc) to the sperm-IPVL assay caused a drastic reduction in the number of IPVL-holes. Therefore, N-linked glycans, possibly with terminal D-GlcNAc residues appear to be involved in sperm-IPVL interaction in chickens. Furthermore, isolated IPVL-glycans were able to induce the AR in chicken spermatozoa.

CHAPTER 1 INTRODUCTION

1. INTRODUCTION

1.1 GENERAL INTRODUCTION

Fertilization involves a series of interactions between spermatozoa and eggs, which ultimately lead to the fusion of gametes and establishment of a new individual. Classical studies of fertilization involved investigation of animals exhibiting external fertilization, particularly marine invertebrates such as the sea urchin (see Longo, 1987). These organisms were studied primarily because they produce a large number of gametes that could be easily fertilized *in vitro*. Early studies of mammalian fertilization proved to be more difficult and challenging, largely due to the fact that fertilization occurs within the female tract and that only a small number of female gametes are available for study. However, the development in the late 1950's, of *in vitro* culture systems that supported mammalian fertilization (Chang, 1959) and their subsequent refinement, made it possible to study the mechanisms of mammalian sperm-egg interaction in the laboratory. As a result, the study of mammalian gamete interaction has been vigorously pursued over the past 40 years or so and considerable progress has been made in understanding the molecular and cellular mechanisms of the mammalian reproductive process.

Generally, molecules involved in a particular biochemical pathway show conservation in structure and function across eukaryotic phyla.

However, many different molecules have evolved in animal phyla to mediate fertilization, suggesting that these molecules may have evolved independently in different animal phyla. Studies of fertilization molecules between and within various animal phyla have been useful for developing our understanding of mechanisms of the fertilization process and for phylogenetic comparisons. In addition to the scientific value of the information obtained, the knowledge gained has been important in manipulating fertilization in many areas, such as agriculture, conservation and medicine.

The field of assisted conception has grown vastly in the last two decades. In humans, the application of *in vitro* fertilization (IVF) and modified IVF techniques, such as gamete intrafallopian transfer (GIFT) has greatly improved the chance of conception in infertile couples, particularly where the problem is due to tubal disease or idiopathic infertility (see Lee, 1991; Edwards and Brody, 1995). More recently methods have been developed to treat severe male infertility problems, including subzonal insemination (SUZI) (see Edwards and Brody, 1995) and intracytoplasmic sperm injection (ICSI) (see Steirtenghem *et al.*, 1995). These techniques circumvent the normal barriers that prevent sperm accessing the oocyte. Viable offspring have also been produced from the transfer of animal embryos produced by *in vitro* maturation (IVM) and IVF, to recipient animals in both domestic (Xu *et al.*, 1987) and non-domestic animals (Donoghue *et al.*, 1990). Efficient methods of IVM and IVF are also important for the development of techniques such as nuclear

transfer and genetic manipulation, which are required for the production of transgenic animals (Hammer *et al.*, 1985).

Although considerable effort has been made in recent years to elucidate the mechanisms involved in fertilization in mammals and to apply this knowledge to improve the reproductive capability of valuable species, very little work has been carried out in birds. This is particularly remarkable as poultry are a globally-important agricultural species. In the United Kingdom alone poultry products are worth approximately £25 million per annum (MAFF UK food and farming in figures, 1997). However, infertility is a considerable problem, particularly with broiler breeders which produce 85% of all poultry and production of fertile eggs in these birds commonly falls below 75% (Wishart and Staines, 1995; Staines *et al.*, 1998). This problem is normally solved by increasing the male to female ratio or to resort to artificial insemination (AI). However, these strategies do not solve the underlying cause and encourage the production of birds with inferior reproductive capabilities.

The first logical step towards improving the reproductive capability of birds is elucidation of the mechanisms involved. Although much work has been carried out on the physiology of egg production and fertility (see Proudman, 1995; Etches, 1995) and, to a lesser extent, sperm production (see Froman, 1995; Howarth, 1995) and storage in the female tract (see Bakst *et al.*, 1994), very little is currently known about the mechanisms involved in the interaction of spermatozoa with the ovum and the AR, an

exocytotic event required for sperm penetration of the ovum, in avian species.

Most of the knowledge gained on reproduction in avian species has come from studies of the domestic fowl. Therefore in this work chickens were used as a model system, although relevant information on other avian species is provided where knowledge is available or necessary. The introduction has been structured to provide a basic understanding of the process of reproduction in chickens. A brief outline of the male and female reproductive tracts are given, encompassing the production of sperm and eggs and the currently available knowledge of the mechanisms involved in fertilization in chickens is given. As most of the information available on the areas studied in this project has come from research on mammalian species, these animals are the logical vertebrate class to draw comparisons from. Therefore, details on mammalian reproductive mechanisms are given alongside those for domestic birds. However, where appropriate, reference will be made to other animal groups.

1.2 AVIAN MALE REPRODUCTIVE PHYSIOLOGY

1.2.1 Spermatogenesis

Spermatogenesis is the differentiation of diploid spermatogonial stem cells into haploid spermatozoa, capable of fertilization. The site of spermatogenesis is the testes, which in birds are found dorsally in the body cavity, close to the kidneys, so that spermatogenisis occurs at the avian body temperature of 40 - 41°C. Each testis is surrounded by a layer of connective tissue and contains an interconnecting network of seminiferous tubules that empty into the rete testis. The epithelium of these tubules is lined with Sertoli cells and germ cells at various stages of development (Lake, 1981; see Froman, 1995). The differentiation of spermatogonia into spermatocytes requires the participation of Sertoli cells, which act as nurse cells enveloping and supporting the developing germ cells. These cells form a continuous layer around the circumference of each tubule and are connected by specialised tight junctions, which form a blood-testis barrier (see Howarth, 1995). In this way substances must first pass through the cytoplasm of the Sertoli cells before being able to contact the developing germ cells (see Froman, 1995). Spermatogenesis is also under hormonal control, through the action of Leydig cells which are found dispersed between the spaces of the seminiferous tubules. In response to stimulation by luteinising hormone (LH), these cells secrete androgens which are thought to act on Sertoli cells to stimulate spermatogenesis (see Howarth, 1995).

In the epithelium of the seminiferous tubules, spermatogonia are constantly produced from stem cells by mitotic division, to give rise to subsequent generations of spermatogonia and primary spermatocytes. In chickens, the primary spermatocytes undergo a prolonged first meiotic division lasting 5.5 days. This results in 2 secondary spermatocytes which subsequently undergo a much shorter second meiotic division (0.5 days) to produce 4 haploid spermatids (Reviers, 1975; see Froman, 1995). During the next 8 days the spermatids continue to elongate without further cell division, resulting in the development of the acrosome, midpiece, flagella and condensation of the nucleus in a process known as spermiogenesis. At each stage of spermatogenesis, the developing germ cell is moved closer to the lumen of the seminiferous tubules where, after 10 - 12 days, it is finally released as a completed spermatozoon (see Lake, 1984).

1.2.2 Sperm Transport and Maturation

Spermatozoa released into the lumen of the seminiferous tubules are swept towards the rete testis in a flow of fluid secreted by the Sertoli cells, before being transported through the excurrent ducts of the male reproductive tract (Figure 1). In chickens this comprises of a short epididymal region, of approximately 20 mm in length and the ductus

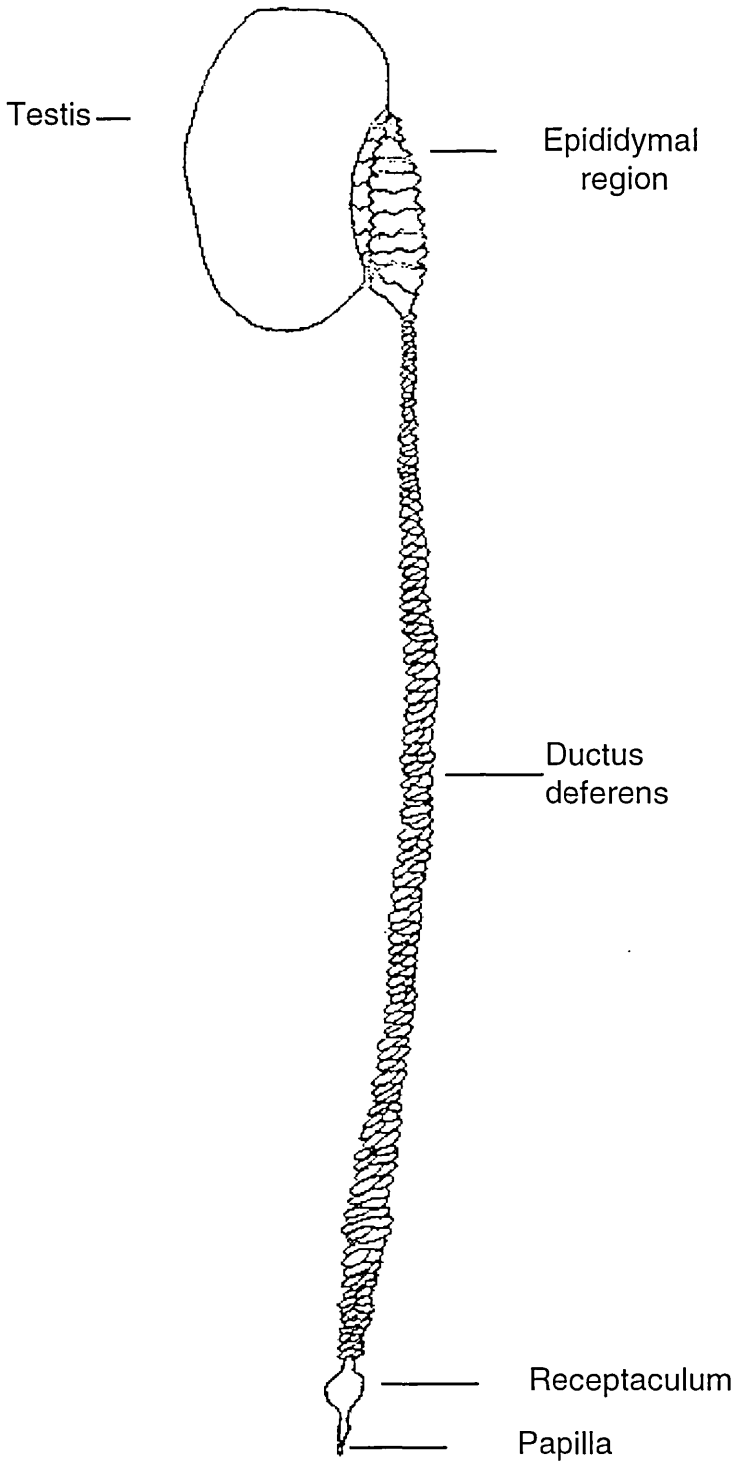


Figure 1. Diagrammatic representation of the reproductive tract of the male chicken showing the testis, epididymal region (comprising the rete testis, ductuli efferentes, connecting ductules and ductus epididymis), ductus deferens, receptaculum and papilla.

deferens, which is a highly convoluted tube of approximately 140 mm in length (Tingari, 1971).

The epididymal region is composed of the rete testis, ductuli efferentes, connecting ductules and the ductus epididymidis (see Froman, 1995). The rete testis is lined with ciliated cells and a small proportion of contractile cells, that are thought to aid the transport of the spermatozoa towards the epididymis (Lake, 1981). Much of the seminiferous fluid is reabsorbed in the rete testis and the ductuli efferentes to give a concentrated suspension of spermatozoa. Transport of spermatozoa through the epididymis and the ductus deferens is largely by fluid flow and the peristaltic movement of the cells of the ducts (Reviers, 1975). The posterior end of the ductus deferens expands into spindle-shaped receptacles which act as the main storage site for spermatozoa (see Froman, 1995). These receptacles penetrate the wall of the cloaca, terminating in short papillae, through which sperm are expelled during ejaculation. In chickens, most of the spermatozoa have been transported from the epididymis within 72 h, depending on the frequency of ejaculation (Reviers, 1975).

In the epididymis, mammalian spermatozoa are known to undergo important maturational changes which are required for motility, the ability to recognise, bind to and ultimately fertilize the ovum (Jones, 1989). Like their mammalian counterparts, avian spermatozoa have also been shown to 'mature' during transport through the excurrent duct of the male tract. In the chicken this maturation involves changes to the sperm surface, including the modification of existing membrane proteins, and the association of proteins and glycoproteins, secreted by cells lining the ducts (Esponda and Bedford, 1985). However, few studies have been carried out on the extragonadal maturation of avian spermatozoa and as a result little is known about the specific functions of these sperm surface associated components.

In contrast to that of mammalian spermatozoa, maturation in avian species does not appear to be directly related to the acquisition of motility or fertilizing ability. Spermatozoa recovered from the testis in chickens are capable of a weak but mature pattern of motility (Munro, 1938; Ashizawa and Sano, 1990) and, although infertile if inseminated intravaginally, can fertilize the ovum if inseminated directly into the magnum (Howarth, 1983).

The sperm-surface characteristics acquired during maturation have been shown to remain associated with the spermatozoa within the oviduct (Morris *et al.*, 1987) and it has been suggested that these proteins may confer upon the sperm the ability to traverse the female reproductive tract (Morris *et al.*, 1987; Esponda and Bedford, 1985). Recently, it has been

shown that removal of sperm surface proteins (Steele and Wishart, 1996a) and terminal sialic acid residues (Steele and Wishart, 1996b) from ejaculated chicken spermatozoa, severely limit the ability of spermatozoa to migrate through the vagina and populate the sperm storage tubules (SSTs). However, if inseminated at the uterovaginal junction these spermatozoa were able to populate the storage tubules (Steele and Wishart, 1996a; 1996b) and gain access to the newly ovulated ovum (Steele and Wishart, 1996a). These experiments give strength to the hypothesis that sperm surface characteristics play an important role in the successful migration of spermatozoa through the vagina, although the mechanism involved is not known. It has been suggested that an immunologically-based selection mechanism may exist in the chicken hen vagina that may impede the progress of spermatozoa with altered surface properties (Steele and Wishart, 1992a).

1.2.3 Semen

In domestic fowl semen is collected by massaging the back of the male, resulting in erection of the phallic structures and papillae due to engorgement by lymphatic fluid. Upon ejaculation, semen is expelled from the papillae into the urodeum of the cloaca. It then flows into the proctodeum between engorged lymphatic folds. The semen is then squeezed into a collecting vessel (Lake and Stewart, 1978; see Froman, 1995).

In avian species there are no secondary sexual organs comparable to those of mammals. However, at ejaculation a transparent, blood-derived, lymph-like fluid can be expressed from the lymphatic folds and may be added to the semen (Nishiyama and Ogawa, 1961; see Fujihara, 1992). The role of this fluid is not known and there is some conflict as to whether it is a normal constituent of semen. Terada *et al.* (1981) suggested that it may provide stimulation for sperm motility. However, Lake (1984) suggested that it may act to reduce the acidity of the surface of the vaginal orifice, caused by contaminating waste material, thereby protecting the spermatozoa deposited there.

Seminal plasma is a complex medium produced as a result of secretion by the epididymis and ductus deferens (Esponda and Bedford, 1985) and selective absorption of components in the efferent ducts (Nakai *et al.*, 1989). The chemical composition differs from blood plasma with respect to electrolyte, amino acid, glucose and protein concentration and composition (see Froman, 1995). It has a total protein content of 8 mg ml^{-1} (Lake, 1966) which is almost 10 times lower than in blood plasma (Blesbois, 1990), and includes a large number of metabolic enzymes, serum-like proteins and proteinase inhibitors. Several free amino acids are found in seminal plasma with glutamate present in very high concentrations (1300 mg ml^{-1}). Considering the very low concentrations of chloride present in seminal plasma, glutamate contributes significantly to the ionic balance of the fluid (see Howarth, 1995). Other known constituents include carnitine, acetyl carnitine, lactic acid, pyruvic acid, ∝-

ketoglutarate, creatine, calcium, sodium, potassium and magnesium (see Lake, 1984) which, with the exception of sodium, all differ in concentration from blood plasma. With respect to sugars, glucose is either absent from seminal plasma uncontaminated with transparent fluid, or present in very low concentrations (see Lake, 1984). Currently, it is not clear how the various components of seminal plasma affect spermatozoa during their passage through the excurrent ducts or after ejaculation.

1.2.4 Spermatozoa

Chicken semen typically contains between 3×10^9 - 8×10^9 spermatozoa ml^{-1} (Lake and Stewart, 1978).

The chicken spermatozoon is a small cylindrical structure with an overall length of approximately 100 µm and approximately 0.5 µm across at the widest point (Lake *et al.*, 1968). Like spermatozoa from other animal species it comprises an acrosome, nucleus, midpiece and flagellum (Figure 2a). The nucleus is a lightly bent cylindrical structure of approximately 12.5 µm in length and is bounded by a double nuclear membrane. The conical shaped acrosome is derived from the Golgi apparatus and measures approximately 2 µm in length. The perforatorium penetrates both the acrosome and the nucleus and may serve to strengthen the links between the two structures. The outer surface of the acrosome is bounded by the outer acrosomal membrane (OAM) and

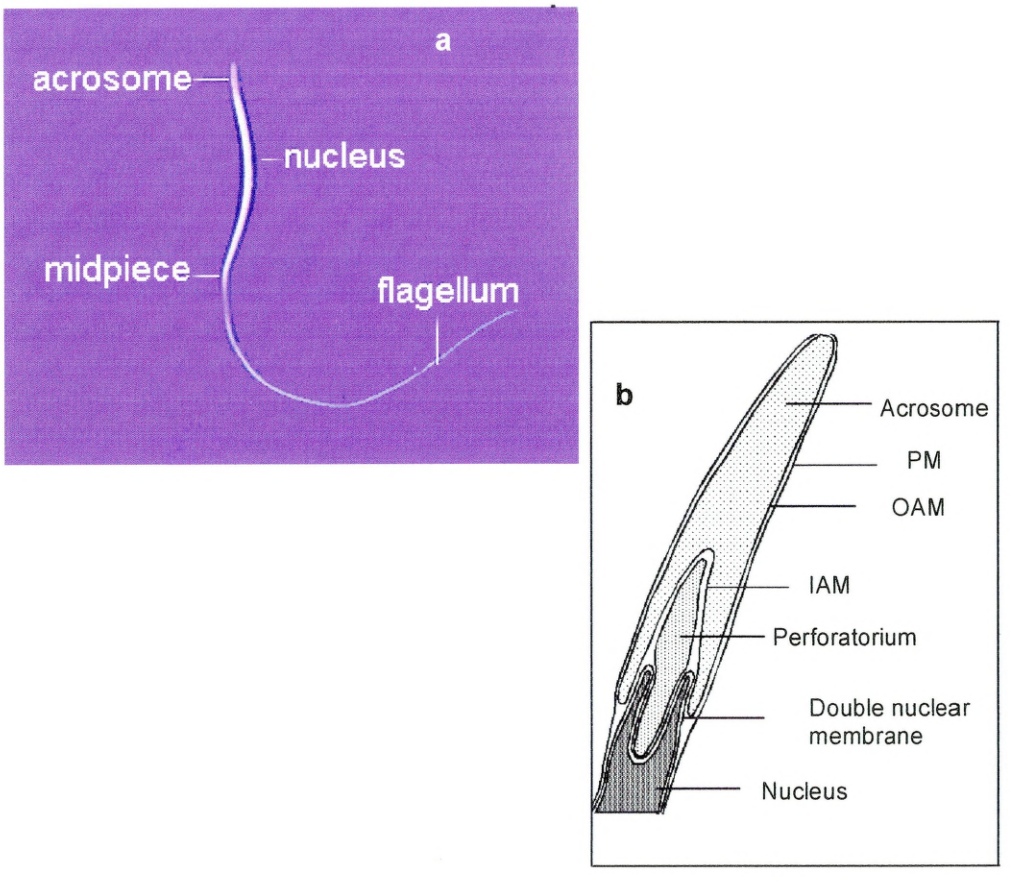


Figure 2. Aniline-eosin smear of a normal chicken spermatozoa (a) depicting the acrosome (length; 2 µm), nucleus (length; 12.5 µm), mitochondria (length; 4 µm) and flagellum (length; 80 µm).The insert (b) shows an enlarged diagrammatic representation of the acrosomal region of the sperm outlining the acrosome, plasma membrane (PM), outer acrosomal membrane (OAM), inner acrosomal membrane (IAM), perforatorium, double nuclear membrane and the nucleus.

appears to be continuous with the inner acrosomal membrane (IAM) which is located adjacent to the perforatorium (see Figure 2b) (Bakst and Howarth, 1975; Thurston and Hess, 1987). The tail, or flagellum, is approximately 80 µm in length and the midpiece, which is approximately 4 µm in length comprises 25 - 30 mitochondria (Bakst and Howarth, 1975; Thurston and Hess, 1987). Under aerobic conditions, these mitochondria provide the enzymatic capability for energy metabolism that enables the tail to flagellate (see Froman, 1995). The entire spermatozoon is surrounded by a plasma membrane which is important for sperm viability and for recognition and binding to the IPVL.

Spermatozoa from different species of domestic birds appear morphologically similar. Turkey spermatozoa are of a similar overall length to those of the chicken, but have an extended midpiece and a shorter, less curved nucleus (Thurston and Hess, 1987). Spermatozoa are more densely packed in ejaculates of turkey semen, which typically contains between 12 - 20 x 10^9 spermatozoa ml^{-1} (Lake and Wishart, 1984). Quail spermatozoa, in comparison, are considerably longer (approximately 140 µm in length) with an extended midpiece comprising of more than 100 mitochondria and a longer, thicker flagellum (Woolley, 1995).

1.3 AVIAN FEMALE REPRODUCTIVE PHYSIOLOGY

The female chicken normally reaches maturity between 16 and 26 weeks (see Burley and Vadehra, 1989). Although the basic timing is under genetic control, several factors influence the onset of sexual maturity, including temperature, nutrition and the duration and intensity of light. Photoperiod, or day length, is the most influential environmental factor and chickens exposed to a short photoperiod during their pre-lay period, followed by an increase in day length display early and synchronised egg laying (see Etches, 1995).

1.3.1 Anatomy of the Female Oviduct

In chickens, as in most birds, only the left ovary and oviduct develop into functional organs (see Gilbert, 1979). The oviduct is approximately 0.6 m long and can be divided into five functionally distinct segments: the infundibulum, magnum, isthmus, uterus and vagina (see Proudman, 1995) (Figure 3).

The infundibulum is approximately 90 mm in length, and is the site of fertilization. The anterior section, or fimbria, is shaped like the open end of a funnel and acts to engulf the newly ovulated ovum. The ovum resides in the fimbria for approximately 15 min (see Etches, 1995; Warren and Scott, 1934), where if spermatozoa are present, fertilization may occur.

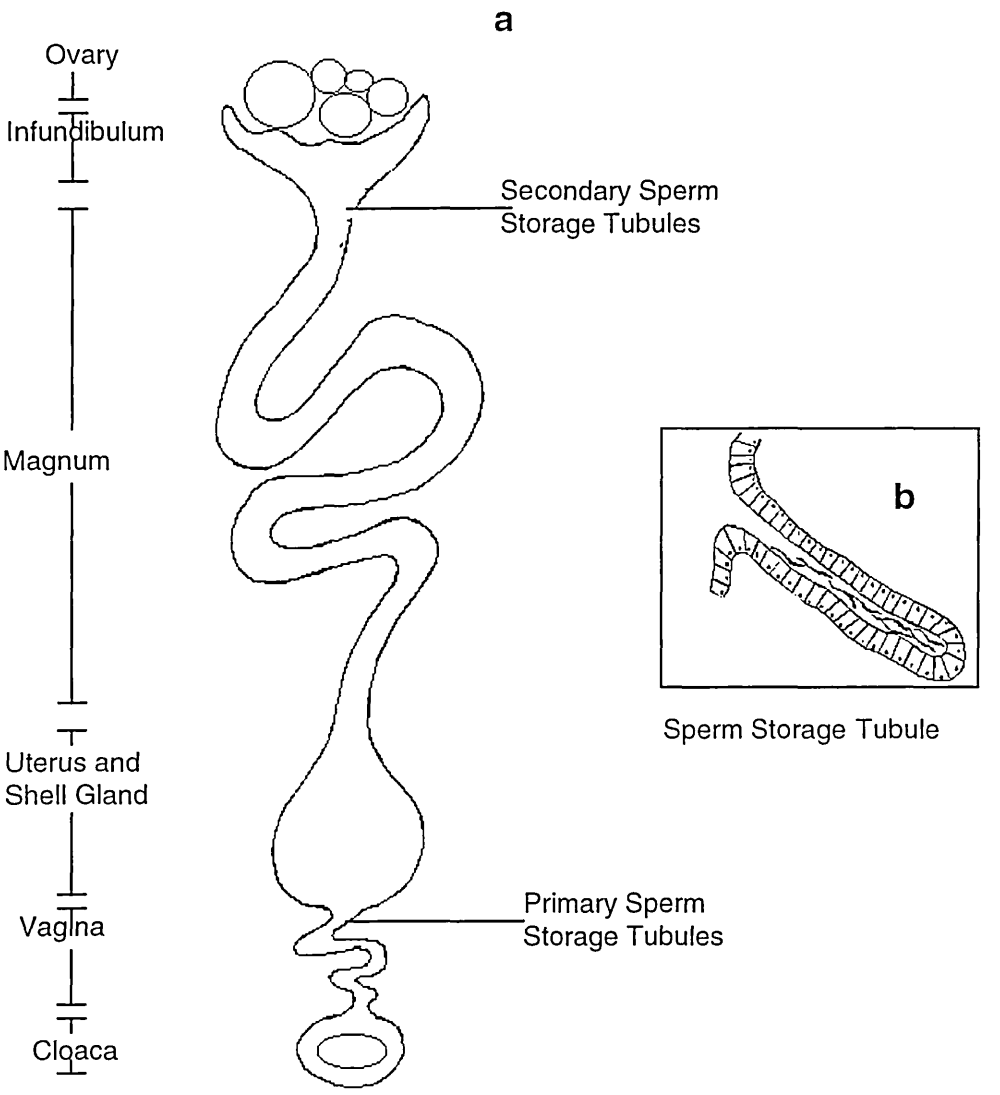


Figure 3. Diagrammatic representation of the reproductive tract of the female chicken (a) showing the ovary, infundibulum, magnum, uterus and shell gland, vagina, cloaca and the primary and secondary sperm storage tubules. Insert (b) shows a diagrammatic representation of a single storage tubule.

Although the muscles of the fimbria are not well developed, contractions increase at the time of ovulation allowing transportation of the ovum through the infundibulum. At the posterior end, the infundibulum converges into a narrow tube and, as the ovum passes through this region, the outer perivitelline layer (OPVL) and the first layer of albumen are laid down (Bakst and Howarth, 1977b). Also located within this region are the secondary sperm storage tubules (Van Drimmelen, 1946) (see Figure 3).

Egg-white albumen proteins are produced in the magnum which, at approximately 320 mm in length, is the longest portion of the oviduct. The ovum remains in this region for approximately 3 h during which time the investment of egg white proteins are laid down around the perivitelline layers. More than 40 different proteins make up the albumen and each has a specific function in embryonic development. Secretion of these proteins is co-ordinated with egg formation and is generally believed to be initiated by mechanical distension of the oviduct as the yolk is transported through the magnum (see Etches, 1995).

After leaving the magnum the egg enters the isthmus, where it resides for approximately 1.5 h. The isthmus is approximately 100 mm long and is the region where the inner and outer shell membranes are deposited around the egg. Both these membranes are composed of protein fibres coated with a glycoprotein matrix, but the fibres of the outer shell membrane are thicker than those of the inner membrane. These

membranes form a final barrier between the developing embryo and the external environment (see Etches, 1995).

From the isthmus, the egg passes into the uterus or shell gland, which is approximately 60 mm in length. The egg remains in the uterus for 18 - 20 h and, during the first few hours, fluid and ions are added to the egg until its mature size is attained, in a process known as 'plumping'. After this initial period the rate of calcification increases while the rate of fluid uptake decreases and the calcified shell is formed (see Proudman, 1995). During the last 2 hours pigments are deposited on the outer surface and the resultant shell is a porous structure through which gas exchange can occur.

Immediately posterior to the uterus and anterior to the vagina lies the uterovaginal junction. Located within this region are the primary sperm storage tubules (Bobr *et al.*, 1964a; Fujii and Tamura, 1963), which are blind ended tubular glands of approximately 2 - 5 mm in width (Lake, 1967, Burke, *et al.*, 1972). The female chicken has approximately 25,000 storage tubules, each of which may contain a few hundred sperm cells. The slow release of sperm from these tubules acts to maintain a high level of fertility over a prolonged period (3 - 4 weeks in chickens and 8 - 15 weeks in turkeys) from a single insemination (Lake, 1975). The mechanisms involved in the release of spermatozoa from these structures is not known.

The vagina is a short, sigmoidal shaped tube of approximately 100 mm in length. It serves as a channel between the uterus and the cloaca, for the fully formed egg to pass through during oviposition. Expulsion of the egg is associated with an increase in intensity of the contractions of the muscles of the shell gland, which occurs immediately before the egg is laid (see Etches, 1995).

1.3.2 Follicular Maturation

The ovary of the domestic laying hen contains oocytes at all stages of development. Each oocyte is enveloped within a follicle, which is composed of a highly vascularised layer of thecal tissue and an inner layer of granulosa cells. The vascularised tissue acts to transport yolk precursors from the liver to the developing follicle, and is extensive in all areas except at the stigma which is the site of rupture at ovulation (see Etches, 1995).

Within the ovary there exists a hierarchy of large yellow yolk follicles from the largest F_1 follicle, that is destined to ovulate first, through to the several thousand smaller follicles from which the large yellow yolk follicles are recruited. The smaller follicles are grouped according to their diameter into the small yellow follicles (6 - 8 mm), the large white follicles (2 - 5 mm) and the small white follicles (< 1 mm). Once a follicle exceeds

8 mm in diameter it is generally recruited into the hierarchy where it continues to accrue yolk until it is ovulated.

The mechanisms involved in maintaining the hierarchy of follicles have not been determined, but the point of control is believed to be at the level of the small yellow follicles (see Proudman, 1995). At 12 -15 weeks these follicles start to produce oestrogens and androgens which initiate the development of the reproductive tract. As the follicles begin to accrue yolk, the production of oestrogen declines and the production of androgens increases (see Etches, 1996). Approximately 16 h after the follicle reaches the F_1 position androgen production is terminated and progesterone production by the granulosa cells increase. This results in a surge in LH which causes dissolution of the stigma on the follicular wall, resulting in release of the ovum at ovulation.

1.3.3 Ovum

At ovulation, the megalecithal chicken ovum is between 30 and 40 mm in diameter. The germinal disc (GD), which is located at the animal pole, is approximately 4 mm in diameter and is identified as a white spot on a background of yellow yolk (Figure 4). Located within this region are the female pronucleus, cell organelles, white yolk spheres, lipid droplets and clear vacuoles (Bakst and Howarth, 1977a). The rest of the ovum lacks organelles and is composed mainly of yellow yolk spheres which provide nutrients for the developing embryo.

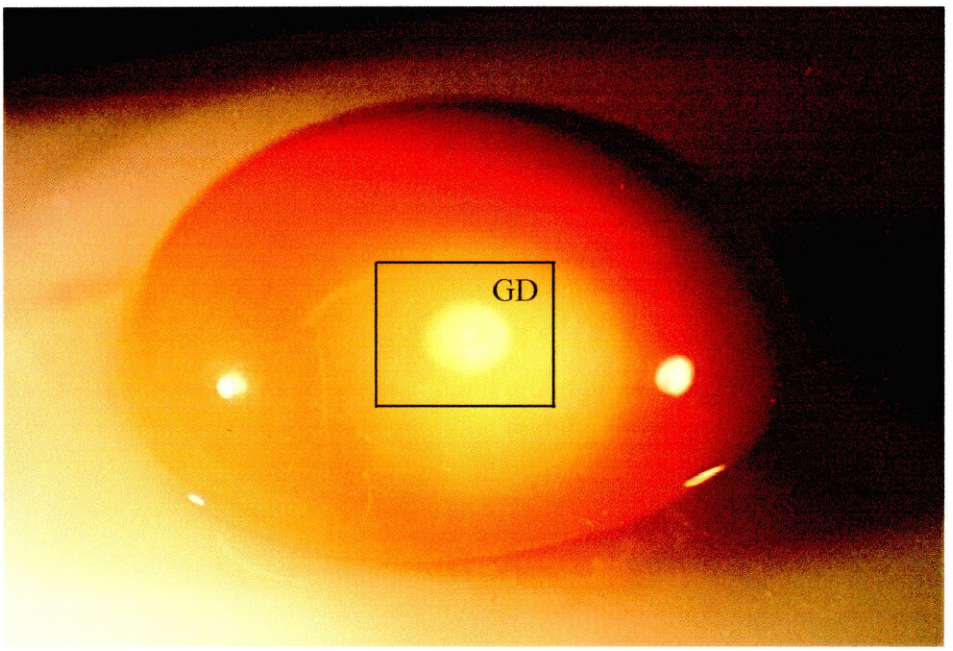


Figure 4. Chicken ovum showing the germinal disc region (GD), located at the animal pole, identified as a white spot against the yellow yolk.

The newly ovulated ovum is bounded by a plasma membrane (oolemma) and the inner perivitelline layer (IPVL). The oolemma is continuous only over the animal pole where it forms a dense array of microvilli which project into the perivitelline space (Bakst and Howarth, 1977a; Bakst, 1978). Surrounding the plasma membrane is the IPVL which provides mechanical support for the megalecithal ovum and presents a barrier to sperm penetration of the ovum and as such can be considered to be analogous to the mammalian zona pellucida (ZP). This layer is between 1 and 3.5 µm thick, consisting of a three-dimensional network of thick cylindrical fibres (Bellairs *et al.*, 1963) and the spaces between these fibres are filled with a granular substance (Bakst and Howarth, 1977b). The fibres overlying the animal pole are thinner and more numerous than in other areas of the ovum (Perry *et al.*, 1978).

Kido *et al.* (1975; 1976; 1977) revealed that the whole perivitelline layer contained three major glycoproteins, designated GPI, GPII and GPIII. GP I and GP II were found to be physically and chemically distinct, but GP II and GP III had the same molecular weight when analysed by reducing SDS-PAGE. Later, Back *et al.* (1982) reported that these components were in fact constituents of the IPVL. GPI was found to be very insoluble and had a molecular weight of 32 kDa by non-reducing SDS-PAGE (Kido *et al.*, 1976; Back *et al.*, 1982). GP II was found to have a molecular weight of 260 kDa under non reducing conditions (Back *et al.*, 1982) and was also found to be hydrophobic in nature. The cDNA for GP1 has been cloned and has been shown to have significant sequence

the ZPC family of proteins (Waclawek *et al.*, 1998; Takeuchi *et al.*, 1999). This protein has been termed gp42 (Takeuchi *et al.*, 1999) or chZP3 (Waclawek *et al.*, 1998) and it has been suggested that this protein is the homologue of the mammalian sperm-binding protein, ZP3 (Waclawek *et al.*, 1998). Glycoproteins equivalent to GP I and GP II have been identified in quail and have molecular weights of 32 kDa and 175 kDa respectively as determined by non-reducing SDS-PAGE. (Mori and Masuda, 1993).

The OPVL is laid down around the IPVL in the upper region of the oviduct approximately 30 min after ovulation (Bellairs *et al.*, 1963; Bain and Hall, 1969; Jensen, 1969). It is composed of a varying number of sublayers, made up of a network of fine fibrils (Jensen, 1969). The layer is between 3 and 8.5 µm thick (Bellairs, *et al.*, 1963; Jensen, 1969) and is composed mainly of lysozyme, ovomucin and vitelline membrane outer proteins I (VMOI) (Back *et al.*, 1982), and II (VMOII) (Kido *et al.*, 1992; see Burley and Vadehra, 1989). The purpose of this layer is thought to be the prevention of further penetration of the IPVL by spermatozoa (Howarth and Digby, 1973; Bakst and Howarth, 1977a).

Electron microscopic studies have shown that between the inner and outer perivitelline layers is a thin membrane of approximately 50 to 100 nm thick (Bellairs *et al.*, 1963). However, this layer has not been isolated or analysed and its function is currently unknown (see Burley and Vadehra, 1989).

1.4 FERTILIZATION

Fertilization is the process by which the spermatozoon and ovum unite, resulting in restoration of the somatic number of chromosomes and the development of a new individual (see Wassarman, 1999). Only when a series of complementary signals and responses have been successfully completed can transfer and syngamy of genetic material occur. In mammals, most of the knowledge gained on gamete interaction and fertilization has come from the study of mice. Therefore, reference will be made principally to the mechanisms involved in fertilization in this species, although reference will be made to other species where appropriate.

1.4.1 Sperm Capacitation

1.4.1.1 Mammals

Following spermatogenesis and epididymal maturation, mammalian spermatozoa are not immediately capable of fertilizing the ovum. Instead an additional period of maturation is required within the female reproductive tract (Austin, 1951; Chang, 1951), or in a 'chemically defined' medium *in vitro* (see Yanagimachi, 1994), where they undergo a series of biochemical and functional modifications required for binding and penetration of the oocyte. These time-dependent changes are collectively called capacitation and result in the development of hyperactivated motility and the ability to undergo the acrosome reaction (AR).

Although the importance of capacitation has been known for several years, the molecular mechanisms underlying these changes are poorly understood. A number of alterations are observed in the plasma membrane of the spermatozoon as a result of capacitation, including removal of the sperm surface-associated glycoprotein coat (Aunomas *et al.*, 1973; Oliphant, 1976); rearrangement of plasma membrane components, including plasma membrane antigens (Myles *et al.*, 1987); a decrease in net negative charge (Rosado *et al.*, 1973); changes in plasma membrane phospholipid composition (Davis *et. al.*, 1980); and changes in lectin binding to the sperm surface (Talbot and Franklin, 1978). Other known changes include increased permeability of the sperm plasma membrane to Ca^{2+} (Singh *et al.*, 1978), increase in adenylate cyclase activity (Mrsny *et al.*, 1984) and activation of acrosomal enzymes (see Clegg, 1983). It has been suggested that the elevated Ca^{2+} levels may regulate the modification of sperm plasma and acrosomal membranes, the activities of acrosin and adenylate cyclase and sperm motility *via* calmodulin (see Longo, 1987).

The site of capacitation appears to vary among species. In species where semen is deposited in the uterus (e.g. pig, dog and many rodents), capacitation occurs mainly in the oviduct. However, in species that deposit semen in the vagina (e.g. sheep, cow and primates), capacitation is initiated there and continues as spermatozoa migrate to the anterior region of the oviduct (see Yanagimachi, 1994). This process does not appear to be strictly species-specific as the female reproductive tract of

one species is capable of capacitating spermatozoa of a different species (see Longo, 1987; see Yanagimachi, 1994).

1.4.1.2 Birds

In domestic birds, capacitation of spermatozoa does not appear to be a necessary requirement for fertilization. Studies have shown that freshly ejaculated chicken spermatozoa inseminated into the vagina following oviposition were able to traverse the female tract and fertilize the ovum within 15 min (Bobr *et al.*, 1964b). Similarly, spermatozoa inseminated directly into the infundibulum just prior to ovulation were also able to fertilize the ovum (Bobr *et al.*, 1964b; Nishiyama *et al.*, 1968). Additionally, *in vitro* studies have shown that chicken spermatozoa (Howarth 1971; Fujihara *et al.*, 1973) and turkey spermatozoa (Howarth and Palmer, 1972) were able to fertilize newly ovulated ova during a 15 min incubation. These results suggest that spermatozoa from domestic birds do not require a period of capacitation in the female tract in order to fertilize ova as they are capable of fertilizing the ovum without a prolonged period in the female tract, or in a capacitation medium *in vitro.*

1.4.2 Sperm-Egg Interaction

1.4.2.1 Mammals

In mammals, sperm-egg interaction is initiated by the binding of spermatozoa to the zona pellucida, which is a unique extracellular matrix surrounding the mammalian egg. *In vitro* studies have shown that this process occurs in two stages (Bleil and Wassarman, 1983). Almost immediately following co-incubation of the gametes, spermatozoa adhere loosely and non-specifically to the zona pellucida in a process known as 'attachment'. In time the contact between the spermatozoa and the zona pellucida becomes more tenacious and binding becomes more specific and irreversible (see Wassarman, 1987).

The mouse egg zona pellucida has been extensively studied and characterised in detail. It is approximately 6.2 µm thick and is composed of three glycoproteins, designated ZP1, ZP2 and ZP3 (Bleil and Wassarman, 1978) of apparent molecular weight of 200 kDa, 120 kDa and 83 kDa respectively. These proteins are arranged in a 3 dimensional lattice work, with ZP2 and ZP3 forming heterodimers, crosslinked by ZP1 (see Wassarman, 1992). The main function of ZP1 is thought to be structural, whereas ZP2 and ZP3 are involved in gamete adhesion. Evidence suggests that ZP3 plays a bifunctional role in gamete interaction; it acts as the primary sperm binding protein (Bleil and Wassarman, 1980) and triggers the AR (Bleil and Wassarman, 1983).

Although acrosome-intact spermatozoa of most mammalian species bind to ZP3, evidence exists to suggest that in some species spermatozoa may undergo the AR prior to zona pellucida binding (Huang, *et al.*, 1981; see Kopf and Gerton, 1991). Following the AR, spermatozoa are thought to interact with ZP2, which acts as a secondary sperm binding protein, maintaining sperm binding to the zona pellucida via their IAM (Bleil and Wassarman, 1986). Spermatozoa are then able to penetrate the zona pellucida, fuse with the plasma membrane of the oocyte and fertilize the ovum.

Fusion of the spermatozoon with the ovum results in 'activation' of the egg and prevention of pathological polyspermy. As a result both ZP3 and ZP2 are inactivated as primary and secondary receptor molecules (see Wassarman, 1992). Modification of ZP3 is thought to be due to the action of a cortical granule glycosidase (Miller *et al.*, 1992; 1994) whereas ZP2 is modified by a cortical granule protease (Gwatkin *et al.*, 1973; Gwatkin and Williams, 1974) resulting in a 'hardening' of the zona pellucida making it more resistant to proteolysis and solubilization (see Wassarman, 1988; see Bleil, 1991).

The proteins on the sperm surface responsible for binding to the zona pellucida have not yet been identified. However, in the mouse, several different sperm proteins have been proposed as possible candidates for the egg binding protein (see Wassarman, 1995). Three of the prominent candidates for primary egg binding are:

sp56, which is found on the heads of acrosome-intact, but not acrosome-reacted spermatozoa (Bleil and Wassarman, 1990);

β-1,4-Galactosyltransferase (Galtase), which is located at the anterior surface of the sperm head plasma membrane (Shur and Hall, 1982) and is thought to aggregate on binding to ZP3, thereby inducing the AR (Macek *et al.*, 1991);

p95, a protein which has tyrosine kinase activity and is thought to be involved in induction of the AR via kinase-activated G-protein-mediated membrane fusion (Burks *et al.*, 1995; see Barros *et al.*, 1996).

Secondary binding to ZP2 has been proposed to be mediated by acrosin in several mammalian species (see Barros *et al.*, 1996).

1.4.2.2 Birds

The IPVL of the avian egg may be considered to be analogous to the mammalian zona pellucida. For fertilization to occur, spermatozoa must first penetrate this layer to gain access to the ovum (Bakst and Howarth, 1977b; Okamura and Nishiyama, 1978a), although currently very little is known about the mechanisms involved. Bellairs *et al.* (1963) and Fujii (1976) suggested that chicken spermatozoa gained access to the ovum by passing through spaces in the fibrous network of the IPVL. However, Bakst and Howarth (1977a) revealed that the apparent 'spaces' between the fibres were in fact filled with a ground substance, particularly on it's outer surface. It is now known that chicken spermatozoa penetrate the IPVL by localised hydrolysis (Ho and Meizel, 1975; Bakst and Howarth, 1977b; Okamura and Nishiyama, 1978a) using acrosomal proteases, including a trypsin-like enzyme (Ho and Meizel, 1970; Howarth and Digby, 1973; Brown and Hartree, 1976).

The female genetic material is located at the GD region and it has been shown that spermatozoa preferentially hydrolyse the IPVL overlying this region (Bramwell and Howarth, 1992a; 1992b; Wishart, 1997) although points of hydrolysis can be found evenly, but sparsely, distributed throughout the layer. The point of entry of each spermatozoon can be identified microscopically as a 'hole' in the layer (Figure 5). A similar situation has also been found in turkey (Wishart, 1997) and quail (Birkhead and Fletcher, 1994).

The reason for the increased hydrolytic activity at the GD is unknown, but several hypotheses have been suggested. These include: chemotactic attraction of spermatozoa to the GD (Howarth and Digby, 1973); the presence of activators of sperm hydrolytic activity (Bakst and Howarth, 1977b); or an increased concentration of receptors in this region (Bramwell and Howarth, 1992b).

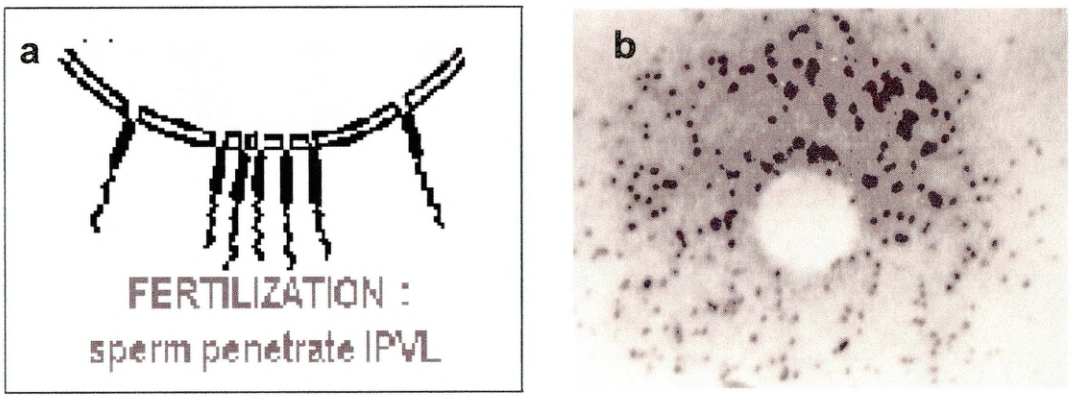


Figure 5. Diagrammatic representation of the preferential hydrolysis of the IPVL overlying the germinal disc by spermatozoa (a) Points of entry of each spermatozoa can be identified as black dots (holes) in the IPVL (b).

Following penetration of the IPVL, spermatozoa enter the perivitelline space where they can contact and penetrate the plasma membrane of the oocyte (Okamura and Nishiyama, 1978b). Although the exact mechanism involved is unknown, two types of sperm entry into the ovum have been proposed. In the first the IAM overlying the perforatorium of the acrosome-less spermatozoon fuses with the oolemma (Okamura and Nishiyama, 1978b) and enters 'head first'. In the second, the

acrosome-intact spermatozoon contacts the oolemma horizontally, penetrates the oocyte plasma membrane and is engulfed by phagocytosis (Okamura and Nishiyama, 1978b; Koyanagi and Nishiyama, 1980; Howarth, 1984). However, Howarth (1984) produced convincing evidence of the microvilli of the oolemma wrapping around the postacrosomal region of the spermatozoon in a manner similar to that found in mammals, suggesting that phagocytic engulfment of acrosome-intact spermatozoa by the oolemma may be the normal mechanism of sperm entry into the ovum.

Although several spermatozoa penetrate into the ovum and form pronuclei, only one is ultimately responsible for fertilization (Fofanova, 1965; Perry, 1987). Approximately 3 h after ovulation, two pronuclei, presumably the male and female pronuclei, occupy the centre of the GD while the remainder move towards the periphery (Harper, 1904; Fofanova, 1965; Perry 1987). Formation of the zygote nucleus occurs approximately 4 h after ovulation and is in mitosis. The supernumerary pronuclei may also undergo one or two rounds of mitosis before disintegrating (Harper, 1904; Fofanova, 1965; Perry 1987).

There appears to be no physiological block to the entry of several spermatozoa into the ovum in birds and it has been suggested that the OPVL may have a role in blocking pathological polyspermy (Howarth and Digby, 1973; Bakst and Howarth, 1977b). The deposition of this layer is thought to provide a mechanical barrier preventing the entry of further

spermatozoa into the IPVL and has also been shown to contain trypsin inhibitors which inhibit the activity of acrosin (Howarth, 1984).

1.4.3 The Role of Carbohydrates in Sperm-Egg Interaction

Glycosylation of proteins is an important post-transcriptional event and glycosylated proteins have been shown to be involved in cell to cell interactions in a variety of systems, including sperm-egg recognition and binding (see Tulsiani *et al.*, 1997). Glycans are attached to proteins through two types of covalent linkages: those that are attached to asparagine residues are known as N-linked glycans; while those attached to serine or threonine residues are known as O-linked glycans. N-linked glycans fall into three categories, (i) complex, (ii) high mannose and (iii) hybrid type sugar chains, which all share the common $Man_3GlcNAc_2$ core sequence. O-linked glycans, in contrast, are structurally diverse, sharing only the anchor GalNAc residue attached to the hydroxl group of serine or threonine (see Paulson, 1989). Extension of these sugar chains commonly results from the addition of $(Gal\beta1,4)_3GlcNAc\beta1,3$ repeat sequence. The addition of terminal sugar residues provides the main source of structural diversity and it is known that terminal glycosylation sequences can vary not only between tissue types, but also between cell types in the same tissue. The huge range of oligosaccharide structures that can arise from glycosylation provides a staggering number of possible

combinations and is consistent with the generation of species-specificity of gamete interactions.

1.4.3.1 Mammals

Each of the 3 glycoproteins found in the mouse zona pellucida have been shown to be heterogeneously glycosylated with both O- and N-linked oligosaccharides. Several lines of evidence strongly suggest that the glycan portion of ZP3 plays an important role in mediating sperm binding to the zona pellucida (see Wassarman, 1999). For example, incubation of gametes with various lectins, monosaccharides, disaccharides, oligosaccharides or glycoconjugates, inhibits sperm-egg interaction *in vitro*, as does treatment of zona intact eggs with exoglycosidases. Furthermore, exposure of purified ZP3 to conditions that alter the conformation of the polypeptide backbone, such as high temperatures, detergents, denaturants or reducing agents fails to abolish the sperm binding activity of ZP3 (see Wassarman, 1995; Tulsiani *et al.*, 1997). However, despite numerous advances, considerable controversy remains regarding the precise identity of the sperm binding moiety of ZP3.

The first direct evidence for the role of carbohydrates in gamete adhesion was produced by Florman and Wassarman (1985) who found that a particular class of O-linked oligosaccharides, of approximately 3.9 kDa, were responsible for the ligand activity of mouse ZP3. However,

several studies have suggested that N-linked glycans may also play a role in sperm-egg binding in mice (Yamagata, 1985; Tulsiani *et al.*, 1989; 1992; Cornwall *et al.*, 1991; Nagdas *et al.*, 1994). Similarly, separate lines of evidence have implicated a role for N-linked glycans (Yonezawa *et al.*, 1995) and O-linked glycans in binding of pig spermatozoa to the zona pellucida (Yurewicz *et al.*, 1991).

Although the structure of the mouse ZP3 oligosaccharide(s) involved in sperm binding have not been identified, evidence suggests that at least one α-linked galactose residue at the non-reducing end of the oligosaccharide chain, is necessary for sperm binding (Bleil and Wassarman, 1988; Johnston *et al.*, 1998). However, transgenic mice lacking in 1,3-α-galactose epitopes were found to be fertile (Thall *et al.*, 1995), suggesting that α-galactose may not be solely responsible for the binding of sperm to ZP3. Further evidence implicates a role for other sugar residues, including mannose (Cornwall *et al.*, 1991), *N*-acetylglucosamine (Miller *et al.*, 1992) and fucose (Johnston *et al.*, 1998) residues. These observations suggest that sperm-egg interaction may be the result of several receptor-ligand interactions between spermatozoa and ZP3 respectively.

Although isolated oligosaccharides and small glycopeptides obtained from ZP3 retain their sperm binding function, they fail to induce the AR (Florman *et al.*, 1984; Florman and Wassarman 1985; Leyton and Saling, 1989), suggesting a role for the polypeptide backbone of ZP3. In

contrast, O-linked glycans have been shown to inhibit sperm-egg interaction in sea urchins (Dhume and Lennarz, 1995), while the AR inducing activity is attributed solely to isolated N-linked glycans (Keller and Vacquier, 1994).

Carbohydrates are also thought to be involved in the loss of ZP3 receptor activity after fertilization, possibly due to the modification of oligosaccharide chains. It has been proposed that cortical granule glycosidases are responsible for the removal of terminal sugar residues from the sperm binding oligosaccharide(s), thereby abolishing sperm recognition (Miller *et al.*, 1992; 1994).

1.4.3.2 Birds

Very little work has been carried out to date on the role of carbohydrates in sperm-egg interaction in avian species. Howarth (1992) demonstrated the importance of perivitelline glycans in gamete interaction, as the removal of both O- and N-linked oligosaccharides from the chicken IPVL abolished sperm binding activity, although the class of glycan responsible for sperm binding was not determined. Recently, Waclawek *et al.*(1998) identified a perivitelline protein of approximately 34 kDa which showed a high degree of homology to mammalian ZP3. This protein was found to contain one putative N-glycosylation site, but as functional

studies were not carried out, the role of this glycan in sperm-egg interaction could not be determined.

1.4.4 Acrosome Reaction

1.4.4.1 Mammals

Following interaction of the gametes, sperm undergo a calcium-dependent, exocytotic event known as the AR. During this reaction the plasma and outer acrosomal membranes fuse at multiple sites (Barros *et al.*, 1967), resulting in the formation of hybrid vesicles and the release of the acrosomal contents.

In all mammalian species studied to-date, successful completion of the AR is an important prerequisite to fertilization. The exact mechanisms involved in this process and the subsequent penetration of the zona pellucida are currently not known. Studies have shown that in most species only acrosome-intact spermatozoa can bind to the zona pellucida, leading to the proposal that the AR occurs after interaction of spermatozoa with the sperm binding ligand of the zona pellucida. However in other species, such as the guinea pig, rabbit and human, both acrosome-reacted and acrosome-intact spermatozoa have been shown to bind to the zona pellucida (see Kopf and Gerton, 1991).

Following induction of the AR, the release of hydrolytic enzymes are thought to digest a thin penetration slit, which, in conjunction with hyperactivated motility, facilitates penetration of the zona pellucida (see Allen and Green, 1997; Bedford, 1998). The obvious candidate for zonal lysis is the serine protease, 'acrosin', which has also been implicated in secondary binding of spermatozoa to the zona pellucida (Jones, *et al.*, 1988). However, the lytic role of acrosomal enzymes has recently been brought into question in eutherian mammals. Current evidence has shown that, although delayed, fertilization still occurs in acrosin-deficient mice, indicating the existence of other enzymes that may facilitate sperm penetration through the zona pellucida (Baba *et al.*, 1994). Further work from the same lab suggests that acrosin plays an important role prior to fertilization in accelerating the dispersal of acrosomal proteins during the AR (Yamagata *et al.*, 1998). Bedford (1998), also questions the lytic role of acrosomal enzymes in eutherian mammals and observed that the AR not only releases hydrolytic enzymes, but changes the apical profile of the sperm head from a blunt, to a tapering edge, favouring penetration dependent on physical thrust. This he suggested, was facilitated by the 'elastic resilient nature' of the zona pellucida.

Following penetration of the zona pellucida, acrosome-reacted spermatozoa are able to gain access to the perivitelline space where they can fuse with the egg plasma membrane, possibly via a sperm protein known as fertilin, and fertilize the ovum.

1.4.4.2 Birds

Little knowledge has been gained on the mechanisms involved in the AR in birds. Originally it was thought that spermatozoa gained access to the ovum through gaps between the fibres of the IPVL (see Section 1.4.2.2, Bellairs *et al.*, 1963). This fitted with the idea that a typical mammalian type AR may not occur in birds and that acrosome-reacted chicken spermatozoa may display only a limited fenestration of the outer acrosomal membrane and some discontinuity of the plasma membrane over the acrosome, resulting in the release of acrosomal enzymes (Bakst and Howarth, 1977b). Furthermore, the identification of hydrolytic enzymes in the avian acrosome (Ho and Meizel, 1970; Howarth and Digby, 1973; Brown and Hartree, 1976), responsible for the localised hydrolysis of the IPVL (Okamura and Nishiyama, 1978a), and the inhibition of IPVL hydrolysis by spermatozoa in the presence of trypsin inhibitors (Howarth and Digby, 1973) suggests that the AR is also a necessary prerequisite to fertilization in birds. A lytic role for acrosomal enzymes is well founded in other species, e.g. sea urchins, amphibian and masupials (see Bedford, 1998), where, as with birds, enzymatic dissociation of the egg investment results in a hole larger than the diameter of the sperm head (Okamura and Nishiyama, 1978a).

Okamura and Nishiyama (1978a), proposed a mechanism for the chicken acrosome reaction that involved the fusion of the plasma and

outer acrosomal membranes, with the subsequent opening of the apical region resulting in the release of lytic enzymes. The acrosome reaction was complete when the acrosome was lost, leaving only the perforatorium surrounded by the inner acrosomal membrane (Figure 6).

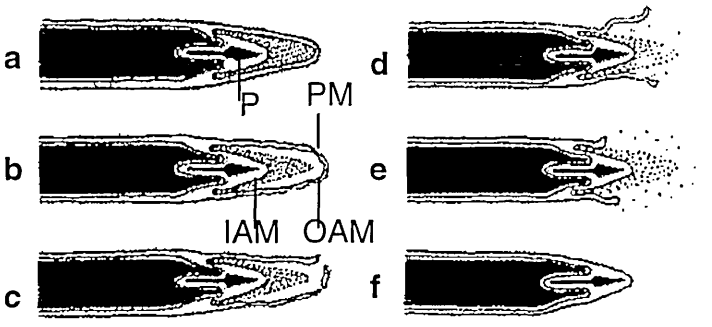


Figure 6. Diagrammatic representation of the AR of the domestic fowl as proposed by Okamura and Nishiyama (1978a): (a) depicts the intact sperm acrosome; (b) the outer acrosomal membrane (OAM) and the plasma membrane (PM) expand and make contact at the apical region; (c) membranes fuse and the acrosome opens at the apical region (d & e);. the membranes are removed releasing the acrosomal contents and finally; (f) the inner acrosomal membrane (IAM) fuses with the plasma membrane to form a continuous membrane around the perforatorium (P).

1.4.5 Interspecies Fertilization

1.4.5.1 Mammals

In mammals, species specificities exist at all levels of the reproductive process, the strictest of which are geographical isolation and the mating behaviour of different species (O'Rand, 1988). As a result of these specificities, fertilization does not normally occur between intact gametes of phylogenetically distant species (Roldan and Yanagimachi, 1989) and fertilization between closely related species generally results in a reduction in the number of fertilized eggs (see Bedford, 1981; Roldan *et al.*, 1985; Maddock and Dawson, 1974). Heterologous combination of gametes are always less successful than homologous combinations (e.g. Roldan *et al.*, 1985; see Yanagimachi, 1994) and the ability of sperm from one species to fuse with the ovum of another does not necessarily mean that the reverse combination will result in fertilization (see Yanagimachi, 1994).

In vivo, the female reproductive tract is thought to play an important role as a barrier to foreign spermatozoa. This could be due to incompatibilities between foreign sperm and the female reproductive tract which may affect factors such as sperm viability, transport to the site of fertilization, or timing of capacitation (see Bedford, 1981). In mammals, barriers to interspecific fertilization have also been shown to occur at both the zona pellucida and the oocyte plasma membrane (Yanagimachi, 1977;

Hanada and Chang, 1978; Bedford, 1981). However, the zona pellucida has been shown to be the primary obstacle to interspecies fertilization at the level of sperm-egg interaction and removal of the zona can allow a wider range of heterologous gamete interactions to occur, including those from distantly related species (Hanada and Chang, 1976; 1978). In some species such as the Chinese hamster, the major block to interspecies fertilization appears to exist, not at the zona pellucida, but at the oocyte plasma membrane (Roldan and Yanagimachi, 1989). In other species, e.g. closely related deer mouse, interspecies fertilization readily occurs (Fukuda *et al.*, 1979) suggesting limited species-specificity at both the zona pellucida and the plasma membrane with these animals.

Carbohydrates have been shown to play an important role in the binding of spermatozoa to the zona pellucida (see section 1.4.3) and it has been suggested that species-specific sugar residues on the zona surface may play an important role in the generation of the species-specificity of gamete interaction (Skutelsky *et al.*, 1994).

1.4.5.2 Birds

Currently very little is known about the barriers to interspecies fertilization in birds, but there is evidence to suggest that incomplete species specificity exists between closely related avian species. Artificial insemination techniques have allowed the production of a small number of hybrids from the insemination of quail hens with chicken semen (Ogasawara and Huang, 1963; McFarquhar and Lake, 1964; Mathis and McDougald, 1987), chicken hens with guinea fowl (Mathis and McDougald, 1987), pheasant hens with chicken semen (Watanabe, 1964) and natural mating of peafowl with guinea fowl (Mathis *et al.*, 1983).

In Aves, barriers to interspecies fertilization is also thought to exist at several levels. However, the only investigation into the role of the IPVL as a barrier to interspecies fertilization was carried out by Bramwell and Howarth (1992c). They showed that there was considerable cross reactivity between heterologous combinations of gametes in chicken, turkey and ducks, suggesting that the IPVL is not a major barrier to interspecies fertilization in birds.

It has previously been suggested that the uterovaginal sperm storage tubules are selective with regard to the spermatozoa that enter them (Allen and Bobr, 1955; Ogasawara et al., 1966) and may therefore play a major role in species-specificity of fertilization in birds. However, foreign spermatozoa are able to efficiently populate the uterovaginal SSTs

of quail (McFarquhar and Lake, 1964) and chicken hens (Steele and Wishart, 1992b) if insemination is carried out at the uterovaginal junction instead of intravaginally. Furthermore, spermatozoa from several avian and mammalian species have been shown to enter the quail uterovaginal junction SSTs *in vitro* (Steele, 1992), indicating that this site is not selective with regard to the spermatozoa that enter them and identifying the vagina as the major site of oviductal sperm selection (Steele and Wishart, 1992b).

1.5 AIMS OF THE PROJECT

1 To investigate the suitability of IPVL, separated from laid chicken eggs, as a substrate for hydrolysis by spermatozoa *in vitro*; and to develop and characterise an *in vitro* assay, utilising the IPVL from laid eggs, based on the frequency of points of hydrolysis (holes) produced in fragments of IPVL.

2. To apply the *in vitro* sperm-IPVL assay, utilising IPVL separated from laid eggs, to assess sperm 'quality' in individual male roosters and in semen that has been stored at low temperature.

3. To investigate the nature of the preferential hydrolysis of the IPVL at the animal pole of chicken ova.

4. To develop a suitable, routine method for assessing the avian acrosome reaction and to use the assay to investigate the regulation of the avian acrosome reaction.

5. To investigate the role of carbohydrates in avian sperm-egg interaction by inhibition of sperm-IPVL interaction through removal of glycans and lectin treatment and by induction of the acrosome reaction with glycans removed from the IPVL.

6. To investigate the efficiency of the IPVL as a barrier to interspecies fertilization in closely and distantly related species.

CHAPTER 2 MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1 MATERIALS

All chemicals were obtained from Sigma Chemical Company Ltd., Poole, Dorset, unless otherwise stated.

2.2 BIRDS AND BIRD MAINTENANCE

Male chickens (*Gallus domesticus)* were from ISA 'Grandparent' cockerels from ISA Poultry Services, Peterborough; chickens hens were ISA Brown commercial layers; male Japanese quail (*Coturnix japonica*) were from a breeding colony at the University of Abertay Dundee. All birds were caged individually, given a photoperiod of 14 h light: 10 h dark and fed a commercial breeder's ration *ad libitum.* Male turkeys (*Meleagris gallopavo*) were from Nicholas Europa Ltd, Kilwinning, and were housed and fed under commercial conditions.

Turkey, duck, pheasant and peahen eggs were obtained from Camperdown Wildlife Park, Dundee; zebra finch eggs were obtained from Professor Tim Birkhead, University of Sheffield.

2.3 SEMEN COLLECTION AND PREPARATION

2.3.1 Chicken Semen

Semen was collected from chickens by abdominal massage as described by Burrows and Quinn (1937). For most experiments, pooled ejaculates from 3 to 4 males were used to provide each sample. The sample was diluted 1:4 in 0.15 mol l^{-1} NaCl with 20 mmol l^{-1} TES (N-Tris[hydroxymethyl]-methyl-2-aminoethanesulfonic acid) at pH 7.4 (NaCl-TES) and stored aerobically in a shaking water bath at 30°C for up to 1 h before use, unless otherwise stated.

2.3.2 Turkey Semen

Semen was collected from turkeys by abdominal massage as described by Burrows and Quinn (1937). Pooled ejaculates from 3 to 4 males were diluted 1:1 in turkey semen diluent at pH 7.1 (Appendix A.2) as described by Lake and Ravie (1982). The diluted semen was placed in 25 ml Nalgene plastic conical flasks and held at 5°C with constant agitation for 2.5 h during transportation to Dundee. Samples were then diluted a further 1:3 in NaCl-TES and stored aerobically at 30°C as described in Section 2.3.1.

2.3.3 Quail Semen

Semen was collected from quails by the method of Ogasawara and Huang (1963) into 100 µl of NaCl-TES. Ejaculates from 2 - 4 males were pooled to provide each sample and incubated aerobically in a shaking water bath at 30°C for up to 1 h before use, as described in Section 2.3.1.

2.3.4 Liquid Storage of Spermatozoa at 5°C Under Aerobic and Anaerobic Conditions

2.3.4.1 Chicken semen

Semen was diluted 1:3 in chicken semen diluent, pH 7.1 (Appendix A.1) as described by Lake and Ravie (1981) and 2 ml samples, contained within 25 ml Nalgene plastic conical flasks, were aerated by agitation in a shaking water bath. Anaerobic conditions were maintained by placing aliquots of diluted semen in sealed 0.5 ml Eppendorf tubes. Both samples were stored for 24 h at 5°C.

2.3.4.2 Turkey semen

Semen was diluted 1:1 in turkey semen diluent, pH 7.1 (Appendix A.2) and transported to Dundee as described in Section 2.3.2, where samples were further diluted 1:3 in turkey diluent and stored as in Section 2.3.4.1.

2.3.5 Determination of Sperm Concentration

2.3.5.1 Haemocytometer counts

Semen samples were diluted 1:1000 in NaCl-TES containing 4% (v/v) formaldehyde, left for 5 min at room temperature, then loaded into both chambers of an improved Neubauer haemocytometer. The grid containing 25 large squares was visualised using a Leitz Labrolux D microscope at x40 magnification and spermatozoa in the four corner and middle squares were counted. This was repeated for the second chamber and the number of spermatozoa ml^{-1} calculated.

2.3.5.2 Colorimetric method

A C075 colorimeter (WPA Ltd., Cambridge) was used to establish a standard curve from which routine sperm concentrations could be derived.

Neat semen was diluted 1:40 in NaCl-TES to give a stock semen sample from which a series of dilutions were made to give sperm concentrations between 1 x 10^6 and 50 x 10^6 spermatozoa ml^{-1}. Sample dilutions were prepared in duplicate, mixed thoroughly and the absorbance read at 550 nm. Aliquots of each dilution were loaded into improved Neubauer haemocytometers and the number of spermatozoa ml^{-1} calculated as in Section 2.3.5.1. The linear regression of optical density at 550 nm to spermatozoa concentration was found to be OD = 0.0241 x -0.0097 (r = 0.99) and from this standard sperm concentrations were established.

2.3.6 Capacitation of Mammalian Spermatozoa

2.3.6.1 Bull spermatozoa

Straws of cryopreserved bull semen were obtained from the Milk Marketing Board Artificial Insemination Centre, New Scone Perthshire. Straws were thawed and diluted 1:25 in Earles's Balanced Salts buffered with 25 mmol l^{-1} HEPES containing 10 mg ml^{-1} bovine serum albumin

(BSA); 2 mmol l^{-1} caffeine and incubated at 37°C under 5% CO_2 for 1 h (W. Ritchie, Roslyn Institute, personal communication).

2.3.6.2 Rat and mouse spermatozoa

Rat and mice were sacrificed by cervical dislocation. The abdomen was soaked in 70% ethanol, cut open and the testis located. One cauda epididymis was removed and suspended in 1 ml of mouse fertilization medium (Appendix B) under oil (Hogan *et al.*, 1994) in a 3.5 cm petri dish. The dish was placed under a dissecting microscope (WILD: Heerbrugg, Switzerland) at x25 magnification and the spermatozoa were gently released into the medium using forceps. After mixing to disperse the sperm cells the dish was incubated at 37°C under 5% CO_2 for 1h.

2.4 SPERM 'QUALITY' ASSAYS

2.4.1 Aniline-Eosin Assessment of Sperm Morphology

The general integrity of the sperm plasma membrane was assessed by examination of aniline-eosin smears as described by Haije (1990). The dye was prepared by vigorously mixing 8% (w/v) aniline blue (water soluble) and 2% (w/v) eosin in '92G' diluent (Appendix A.3) for 1 h. The remaining insoluble material was removed by centrifugation (IEC Centra-4R; International Equipment Company, Bedfordshire) at 700 g for 10 min. The supernatant was removed and stored in 1 ml aliquots at -20°C until required.

A 1:30 dilution of sperm suspension was prepared, mixed thoroughly and incubated at 5°C for 2 min. A volume of 20 µl was smeared onto a microscope slide, dried immediately in a stream of cold air and examined microscopically at x1000 magnification. Slides were prepared in triplicate for each assay and a minimum of 100 spermatozoa were counted per sample. The percentage of spermatozoa that showed normal morphology and was impermeable to eosin was calculated.

2.4.2 Sperm Tetrazolium Dye Reduction Assay

The ability of spermatozoa to reduce the colourless tetrazolium salt 2-[*p*-iodophenyl]-3-[*p*-nitrophenyl]-5-phenyltetrazolium chloride (INT) to a strongly coloured formazan was measured as described by Chaudhuri and Wishart (1988). Stock solutions were brought to room temperature and all assay constituents (Appendix C.1) were combined in 75 mm polycarbonate test-tubes. A 40 µl aliquot of semen was added to each tube, mixed thoroughly and the mixture incubated in the dark at room temperature for 20 min. The reaction was stopped by the addition of 200 µl of 5% (v/v) Triton X-100 in 0.1 mol l^{-1} HCl mixed and allowed to stand, in the dark, at room temperature for 5 min. Samples were then centrifuged at 700 g for 10 min and the absorbance of the supernatant measured spectrophotometrically (LKB NOVASPEC II, Biochrom, Cambridge) at 520 nm. Assays were carried out in triplicate and the reduction capacity of the samples were calculated as nmoles INT-formazan produced per hour per billion sperm using the molar extinction coefficient of INT-formazan at 520 nm = 15.5×10^3 (Chaudhuri and Wishart, 1988).

2.4.3 Assessment of Sperm ATP Concentration

Sperm ATP concentration was measured in boiled extracts with firefly luciferase after the method of Wishart (1982). Aliquots of 200 µl of distilled water were added to glass boiling tubes and boiled for 2 min in a boiling bath, before the addition of 100 µl of sperm suspension. After a further 2 min incubation, the tubes were removed from the boiling bath, cooled to room temperature and centrifuged at 700 g for 5 min. The supernatants were removed and assayed for ATP content. A stock solution of firefly lantern extract was diluted 1:15 with distilled water and 800 µl loaded into a 1 ml syringe and added to 100 µl of boiled extract, or 10 µl of 10 µmol l^{-1} ATP standard, in a luminometer (Bio Orbit 1250. LKB Wallace, Turku, Finland). An output was obtained on a connected Servigor chart recorder from which the ATP concentrations could be calculated.

2.4.4 Subjective Assessment of Sperm Motility by Light Microscopy

Semen samples were diluted 1:400 in NaCl-TES and a 10 µl aliquot placed onto the centre of a microscope slide. A coverslip was place over the sample and the slide viewed at x40 magnification. Motility was scored between 0 and 5, with 0 representing samples with virtually no motility and 5 representing samples where motion was vigorous.

2.5 PREPARATION OF PERIVITELLINE LAYERS

2.5.1 Isolation of IPVL from Ovulated Ova

Chicken hens were sacrificed by injection of 3 ml Euthatal (RBM Animal Health Ltd, Dagenham) into the wing vein, approximately 15 minutes after oviposition. The abdomen was opened and the newly ovulated ovum was collected from the body cavity or first few centimetres of the infundibulum. The ovum was suspended in 1% (w/v) NaCl, cut equatorially until almost in two and the inner perivitelline layer 'peeled' from the yolk. The isolated layer was washed free of adherent yolk by several washes in 1% (w/v) NaCl and was finally suspended in NaCl-TES and stored at 5°C for up to 24 h.

2.5.2 Isolation of IPVL from Follicular Oocytes

Follicular oocytes were removed from the ovaries of birds sacrificed as described in Section 2.5.1. Follicles were cut open and washed in 1% (w/v) NaCl to remove adhering yolk. The IPVL was gently teased from the follicle wall using forceps, washed thoroughly in several changes of 1% (w/v) NaCl and finally rinsed in distilled water to remove the granulosa cells. Isolated IPVL was stored in NaCl-TES at 5°C for up to 24 h.

2.5.3 Isolation of Whole Perivitelline Layer from Laid Eggs

Freshly laid unfertilised eggs were cracked open, the yolks isolated and washed free of albumen. The isolated yolks were cut open and washed in several changes of 1% (w/v) NaCl to remove adhering material and the perivitelline layers stored in NaCl-TES at 5°C for up to 24 h before use.

2.5.4 Separation of the Inner and Outer Perivitelline Layers of Laid Eggs

In eggs from all species, except zebra finch, the IPVL was separated from the OPVL of laid eggs by acid hydrolysis as described by Kido and Doi (1988). Freshly laid, unfertilised, eggs were cracked open and the yolks isolated, then washed free of albumen in several washes of 1% (w/v) NaCl. Stubborn pieces of albumen were removed using forceps. Yolks were then immersed in 100 ml beakers containing approximately 75 ml of 0.01 mol l^{-1} HCl and incubated at 37°C for 1 h. After incubation the HCl was removed and the yolk decanted into a dish containing 1% (w/v) NaCl. The yolks were punctured and the perivitelline layer washed several times in 1% (w/v) NaCl to remove adhering yolk material. The perivitelline layer was spread out in a petri dish containing 1% (w/v) NaCl and the inner and outer layers carefully pulled apart using forceps. The isolated IPVL was stored at 5°C for up to 24 h.

Zebra finch egg shells were carefully cut open, and the yolks isolated and washed free of albumen as before. The isolated yolks were subsequently immersed in 1% (w/v) NaCl, and the perivitelline layer cut open until almost in two. The layer was washed free of adhering yolk material by several washes in 1% (w/v) NaCl and placed in a 10 cm petri dish containing fresh 1% (w/v) NaCl. The dish was placed under a dissecting microscope (WILD: Heerbrugg, Switzerland) at x25 magnification and the IPVL was simply pulled apart from the OPVL using forceps. The isolated inner layers was stored at 5°C for up to 24 h.

2.5.5 FITC-Conjugated Lectin Labelling of IPVL

Stock solutions of 1 mg ml^{-1} were prepared in NaCl-TES for lectins from Peanut agglutinin (PNA), *Pisum sativum* agglutinin (PSA), *Solanum tuberosum* agglutinin (STA), *Tetragonolobus purpureas* agglutinin (LTA) wheat germ agglutinin (WGA) and *Ulex europaeus* agglutinin *II* (UEA II). Concanavalin A (Con A) was prepared in NaCl-TES containing 0.1 mmol l^{-1} $CaCl_2$ and 0.1 mmol l^{-1} $MnCl_2$ (see Appendix D for list of lectin specificities).

IPVL was separated from laid chicken eggs as described in Section 2.5.4. In each experiment IPVL from the same laid egg was used. Pieces of IPVL of approximately 0.5 cm x 0.5 cm were excised and added to 30 ml polycarbonate vials, containing 1 ml of fluorescein isothiocyanate

Vials were incubated at room temerature in light-shielded containers for 30 min. The IPVL was washed several times in NaCl-TES to remove unbound lectin, spread onto microscope slides and covered with a coverslip. The slides were examined by epifluorescence at x400 and x1000 magnification and the degree of fluorescence noted on a subjective scale of 0 to XXXXX, where 0 represents no fluorescence and XXXXX represents intense fluorescence.

2.6 SPERMATOZOA-PERIVITELLINE LAYER INTERACTION

2.6.1 Assessment of Spermatozoa-IPVL Interaction and Fertility *In Vivo*

Groups of 5 hens were inseminated intravaginally with undiluted semen containing approximately 60 x 10^6 spermatozoa on 3 occasions, each 2 days apart. The eggs laid between days 2 and 7 were collected and examined for fertility by the method of Kosin (1944). Essentially, eggs were cracked open and the albumin removed by decanting the yolk repeatedly from one half of the egg shell to the other. The isolated yolk was placed into a small dish containing 1% (w/v) NaCl and the GD region located. Blastoderms were examined to determine if the eggs were fertile. Intact, circular, blastoderms were scored as fertile and incomplete blastoderms containing visible vacuoles were scored as infertile.

Pieces of PVL, approximately 1 cm^2 in size, were cut from GD and non-GD regions of each egg and washed several times in 1% (w/v) NaCl to remove adhering yolk and albumin. The PVLs were carefully spread onto a microscope slide and covered with a coverslip. Slides were examined at x40 magnification using darkground optics. The total number of holes in the IPVL over the GD region were counted. For areas away from this region the number of holes per microscope field (16 mm^2) were counted and the number of holes per mm^2 calculated.

2.6.2 *In Vitro* Fertilization of Ovulated Chicken Ova

Ovulated chicken ova were obtained from chicken hens approximately 15 min after oviposition as described in Section 2.5.1. and incubated in 100 ml beakers containing 1.25 x 10^7 spermatozoa ml^{-1} in a total volume of 30 ml Dulbecco's Modified Eagles' Minimal Essential Medium, buffered with 10 mmol l^{-1} HEPES (DMEM) (GIBCO BRL, Life Technologies, Paisley U.K.) for 5 min at 40°C. The IPVL overlying the GD, and pieces of non-GD IPVL (approximately 0.5 cm x 0.5 cm), were removed from fertilized ova (see Section 2.6.1), washed several times in 1% (w/v) NaCl and either spread onto microscopes slides and examined as in Section 2.6.1, or incubated in the *in vitro* sperm-IPVL assay as described in Section 2.6.3.

2.6.3 Spermatozoa-Perivitelline Layer Interaction Assay *In Vitro*

Pieces of IPVL, of approximately 0.5 cm x 0.5 cm were incubated with spermatozoa at 40°C in 30 ml polycarbonate vials containing a final volume of 1.0 ml DMEM. Standard assays employed sperm concentrations of 1.25 x 10^7 spermatozoa ml^{-1} and a 5 min incubation period (Steele *et al.,* 1994), although these conditions were varied experimentally. Variations in sperm concentrations and assay times used are noted in the appropriate results section. After incubation, the pieces of IPVL were removed from the vials and washed in NaCl-TES to remove

loosely adhering spermatozoa. The IPVL pieces were spread onto microscope slides, covered with a coverslip and examined at x100 magnification using darkground optics. The number of IPVL-holes were counted in videoprints of 3 fields of view, each of 0.55 mm^2 and the number of holes per mm^2 calculated

2.6.3.1 Assessment of preferential binding of spermatozoa to the inner or outer IPVL

A specially prepared perspex block was prepared containing 2 cavities of approximately 5 mm in diameter (Figure 7). To each cavity 120 µl of DMEM containing 1.25 x 10^7 spermatozoa ml^{-1} was added. IPVL from laid eggs of approximately 2 cm x 2 cm were cut and spread out in a petri dish containing NaCl-TES. The IPVL piece was cut in 2 and 1 piece was placed over 1 of the cavities on the perspex slide. The remaining piece of IPVL was flipped over and placed over the second cavity, thus ensuring that both the inner and the outer surface of the IPVL were in contact with the spermatozoa. The slide was incubated at 40°C for 5 min, after which the IPVL was removed and washed briefly in NaCl-TES. The IPVL was spread on a microscope slide, covered with a coverslip, viewed at x100 magnification and the number of holes per mm^2 calculated as in Section 2.6.3. The IPVL was then viewed under x1000 magnification to determine if spermatozoa were exposed to the inner or the outer surface of the IPVL.

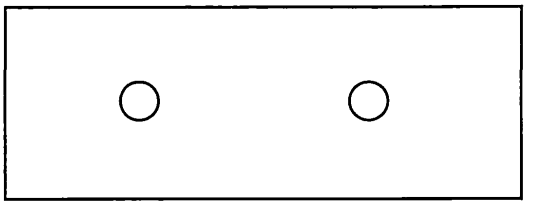


Figure 7. Diagrammatic representation of perspex block (not to scale) containing two cavities, each 5 mm in diameter, used to determine the preferential hydrolysis of spermatozoa to the inner or outer surface of the IPVL.

2.6.3.2 Assessment of role the of egg yolk in the inhibition of hydrolysis of the IPVL by spermatozoa

Ovulated ova were obtained from the body cavity of chickens as in Section 2.5.1. IPVL pieces of approximately 0.5 cm x 0.5 cm were excised from the ovum, scooped out taking care to retain adhering yolk material, then placed in a 3.5 cm petri dish to which 1.25×10^7 spermatozoa ml^{-1} was carefully added to a final volume of 3 ml in DMEM. Care was taken not to dislodge the IPVL. The dish was incubated, unshaken at 40°C for 5 min. The IPVL was removed, washed several times in NaCl-TES and the number of holes per mm^2 calculated as in Section 2.6.3. Control samples were washed free of adhering yolk and incubated under the same conditions.

Pieces of IPVL of approximately 0.5 cm x 0.5 cm were removed from the same piece of ovulated IPVL and incubated with 10% yolk in DMEM in the *in vitro* sperm-IPVL assay as described above without shaking and the number of holes per mm^2 calculated.

2.6.3.3 The effect of saccharides on sperm-IPVL interaction *in vitro*

A range of saccharides were added to the *in vitro* sperm-IPVL assay (see Section 2.6.3 for protocol) to determine their effect on hydrolysis of the IPVL by spermatozoa. Monosaccharides, D-glucose (D-glu), D-galactose (D-gal), D-fucose (D-fuc), L-fucose (L-fuc) and D-mannose (D-man) were added at 0.1 mol l^{-1}, and D-N-acetylglucosamine (D-GlcNAc) at 0.75 mol l^{-1}. Fucoidin was added at 1 mg ml^{-1} and 0.1 mg ml^{-1}; *N-N-* diacetylchitobiose (Fluka) was added at concentrations of 0.2 mmol l^{-1} and 0.04 mmol l^{-1}.

2.6.3.4 The effect of Lectins on sperm-IPVL interaction *in vitro*

Stock solutions of 1 mg ml^{-1} were prepared in NaCl-TES for all lectins used (see Appendix D for list of lectins used and lectin specificities), with the exception of Con A which was prepared as described in Section 2.5.5.

Pieces of IPVL separated from laid eggs (see Section 2.5.4 for protocol), of approximately 0.5 cm x 0.5 cm, were added to 30 ml polycarbonate vials containing 1 ml of either 10 µg ml^{-1} or 100 µg ml^{-1} lectin. Vials were incubated for 4 h at room temperature, then washed thoroughly in NaCl-TES to remove unbound lectin. The IPVL pieces were incubated in the *in vitro* sperm-IPVL assay as in Section 2.6.3 and the number of holes per mm^2 calculated.

2.6.3.5 Determination of the ability of WGA to inhibit hydrolysis of the IPVL by spermatozoa in the presence of *N,N'*-diacetylchitobiose

A stock solution of 100 mmol *N,N'*-diacetylchitobiose l^{-1} was prepared in NaCl-TES and stored at -20°C until required. Polycarbonate vials containing either 100 µg ml^{-1} WGA, or 100 µg ml^{-1} WGA in the presence of *N,N'*-diacetylchitobiose at concentrations of 5 mmol l^{-1},

1 mmol l^{-1}, 0.2 mmol l^{-1} or 0.04 mmol l^{-1} were incubated for 30 min at room temperature. Pieces of IPVL separated from laid eggs, of approximately 0.5 cm x 0.5 cm, were added to each vial and incubated for a further 4 h at room temperature. The IPVL pieces were then removed from the vials and washed several times in NaCl-TES to remove unbound lectin and sugar, then incubated in the *in vitro* sperm-IPVL assay as in Section 2.6.3 and the number of holes per mm^2 calculated.

2.7 ACROSOME REACTION ASSAYS

2.7.1 Methods Of Exposing Acrosomal Membranes

2.7.1.1 A23187

A stock solution of 5 mmol l^{-1} A23187 was prepared in dimethylsulfoxide (DMSO) and stored in 10 µl aliquots at -20°C. Immediately before use an aliquot was thawed and 90 µl of DMEM added. Spermatozoa samples, diluted 1:25 for bull and 1:50 for chicken, were microcentrifuged at 1889 g for 1 min and the pellet resuspended in 10 µmol l^{-1} A23187 in DMEM. Samples were incubated at 37°C under 5% CO_2 in air for 5 min to 1 h.

2.7.1.2 Sonication

Spermatozoa samples were diluted 1:40 in NaCl-TES and sonicated for either 1, 2 or 3 second pulses at output 1 using a Sonics and Materials Vibra Cell Sonicator, Connecticut, USA.

2.7.1.3 Detergent treatment

Spermatozoa samples were diluted 1:20 and incubated for 5 min in either 0, 10, 25, 50 75 or 100 μg ml^{-1} digitonin. Following incubation the samples were microcentrifuged at 1889 g for 1 min.

2.7.2 Detection Of The Avian Acrosome Reaction

2.7.2.1 Simple microscopic methods

IPVL from a laid chicken egg was homogenised in 10 ml of DMEM and aliquots of 1 ml of the IPVL preparation were incubated with 1.25 x 10^7 spermatozoa ml^{-1} for 5 min at 40°C. Following incubation the samples were microcentrifuged at 1889 g for 30 sec. The resultant sperm pellet was resuspended in 50 μl of NaCl-TES and a 10μl aliquot was placed on a microscope slide, covered with a coverslip and examined by phase

contrast or differential interference microscopy at x1000 magnification for modification of the acrosome.

2.7.2.2 Chlortetracycline labelling

Chlortetracycline (CTC) solution containing 500 µmol l^{-1} CTC; 5 mmol l^{-1} L-cysteine in NaCl-TES was prepared fresh and stored at 4°C in a light shielded container before use.

The method used was a modification of that described by DasGupta *et al.*, 1993. An aliquot of sperm containing 1.25 x 10^7 spermatozoa ml^{-1}, with either exposed acrosomes or control samples, in a final volume of 1 ml, was microcentrifuged at 1889 g for 1 min. The resultant pellet was resuspended in 50 µl of NaCl-TES in a foil wrapped microcentrifuge tube, to which 50 µl of CTC solution was added and mixed thoroughly. Cells were fixed by the addition of 5 µl of 12.5% (v/v) glutaraldehyde (grade 1) in NaCl-TES, mixed thoroughly and an 8 µl aliquot placed on a microscope slide. A coverslip was placed on top of the sample and the slide was gently compressed between tissues to remove excess fluid. Slides were examined immediately using epifluoresence microscopy (excitation = 395 nm; emission = 510 nm). In all experiments a minimum of 100 sperm were counted.

2.7.2.3 Lectin staining of avian sperm acrosomes

2.7.2.3.1 *FITC-lectin labelling of spermatozoa*

Stock solutions of 1 mg ml^{-1} in NaCl-TES were prepared for lectins from WGA, LTA, PSA and PNA, and in NaCl-TES containing 0.1 mmol l^{-1} $CaCl_2$ and 0.1 mmol l^{-1} $MnCl_2$ for Con A (see Appendix D for lectin specificities).

The protocol for spermatozoa staining was essentially that described by Cross and Overstreet (1987) for unfixed human spermatozoa. Samples containing 1.25 x 10^7 spermatozoa ml^{-1} with either exposed acrosomes or control samples, in a final volume of 1 ml, were microcentrifuged at 1889 g for 1 min. The pellet was resuspended in 45 µl of NaCl-TES to which a 5 µl aliquot of lectin was added to give a final concentration of 100 µg ml^{-1}. Samples were incubated at room temperature for 10 mins in light shielded containers, washed in 1 ml of NaCl-TES and microcentrifuged at 1889 g for 1 min. The pellet was resuspended in 50 µl of NaCl-TES and a 10 µl aliquot was placed on a microscope slide, covered with a coverslip and examined by epifluorescence microscopy at x1000 magnification for alterations in staining patterns. A minimum of 100 spermatozoa were counted in each experiment.

2.7.2.3.2 *Colloidal gold - PNA labelling of spermatozoa for transmission electron microscopy (TEM)*

Colloidal gold conjugated-PNA was diluted to a working concentration of 100 µg ml^{-1} in NaCl-TES with 0.5% Albumin and 0.05% Tween 20. Chicken spermatozoa with both intact and exposed acrosomes were prepared and labelled with colloidal gold conjugated-PNA following the method described in Section 2.7.2.3.1. Following incubation at room temperature for 15 min in a light shielded container, samples were microcentrifuged at 1889 g for l min and fixed in 2.5% glutaraldehyde (grade 1) in 0.1 mol l^{-1} cacodylate buffer. Samples were processed for TEM studies by Dr.Lena Holm at the Swedish University of Agricultural Sciences, Uppsala, Sweden.

2.7.3 Determination of the Effect of Calcium and Temperature on the Acrosome Reaction in Chicken Spermatozoa

IPVL from 1 laid chicken egg was homogenised in 10 ml of NaCl-TES containing either 5 mmol $l^{-1}Ca^{2+}$, or 1 mmol l^{-1} EGTA to remove extracellular Ca^{2+}. Spermatozoa was added to a final concentration of 1.25×10^7 cells ml^{-1} and incubated at either 30°C or 40°C for 5 min. The acrosomal status of spermatozoa was determined by FITC-PNA labelling of exposed acrosomes as described in Section 2.7.2.3.1.

2.8 FRACTIONATION OF IPVL AND ANALYSIS OF ACROSOME REACTION INDUCING MOIETIES

2.8.1 Fast Protein Liquid Chromatography (FPLC) Separation of Solubilised IPVL Proteins and Analysis of Acrosome Reaction Inducing Activity

IPVL was separated from laid chicken eggs as described in Section 2.5.4. Each IPVL was solubilised in 10 ml of 5 mmol l^{-1} NaH_2PO_4; pH 2.5 and incubated for 1 hr at 70°C. Samples were centrifuged at 3000 g for 15 min and the supernatant retained.

FPLC protein analysis was carried out to achieve high resolution separation of the solubilised IPVL proteins. A superdex gel filtration column was equilibrated overnight in 0.85% NaCl, 500 µl aliquots of the solubilised protein sample were injected onto the column using a flow rate of 0.5 ml min^{-1} with a chart speed of 0.5 cm min^{-1} and fractions of 1 ml were collected. Samples corresponding to the peaks were immediately incubated with 1.25 x 10^7 spermatozoa ml^{-1} at 30°C for 5 min (see Section 2.7.4 for method) and assessed for acrosome reaction inducing activity using FITC-PNA lectin labelling of exposed acrosomes (see Section 2.7.2.3.1 for method).

2.8.2 Chemical Cleavage of O- and N- Linked Glycans from IPVL and Analysis of Acrosome Reaction Inducing Activity of Released Glycans

Liberation of O- and N-linked glycans can be achieved by the successive use of reductive β-elimination and alkaline cleavage (Montreuil *et al.*, 1994). O-glycosidic linkages between glycans and serine and thereonine residues on the polypeptide backbone are easily cleaved by the action of mild alkali (β-elimination). N-glycosidic linkages are stable under these conditions and require more drastic alkaline conditions for liberation.

2.8.2.1 Successive β-elimination and alkaline cleavage

IPVL separated from 10 laid eggs (see Section 2.5.4 for protocol) was added to a 100 ml round bottomed flask to which 10 ml of 1 mol l^{-1} $NaBH_4$-0.05 mol l^{-1} NaOH was added and heated for 16 h at 45°C in order to cleave O-linked glycans. Following this, N-linked glycans were liberated by the addition of 10 ml of 1 mol l^{-1} $NaBH_4$ -2 mol l^{-1} NaOH to the flask and heated under reflux for 6 h at 100°C. The solution was cooled, placed on ice and neutralised by the addition of 50% (v/v) acetic acid to a pH of 6. The solution was added to a 50 ml sterile centrifuge tube and centrifuged at 3000 g for 10 min and the supernatant retained.

2.8.2.2 Purification of glycan fractions by gel filtration

The supernatant obtained in Section 2.8.2.1 was filtered and purified on a Sephadex G25 column (40 x 2.5 cm), prepared and eluted in distilled water. The column was run at 2 ml min^{-1} and fractions of 8 ml were collected. Aliquots of 1 ml were removed from each fraction and assessed for the presence of total sugar, using the orcinol sugar assay (see Section 2.10) and protein, using the bicinchoninic acid (BCA) protein assay (see Section 2.11).

The glycan containing fractions were pooled and lyophilised overnight. The lyophilised glycans were re-*N*-acetylated by dissolving the sugar in 5 ml saturated sodium bicarbonate ($NaHCO_3$), before the addition of 7 x 10 µl aliquots of acetic anhydride at 5 min intervals. The remaining precipitate was centrifuged at 3000 g for 10 min, the supernatant removed and desalted on a H^+ amberlite column (5 cm x 1 cm). The eluant was collected in 2 ml fractions and assessed for sugar content (see Section 2.10), pooled and lyophylised as before.

2.8.2.3. Analysis of the acrosome reaction inducing activity in IPVL glycan fractions

O- and N-linked glycans were removed from the IPVL of laid chicken eggs as described in Section 2.8.2. The lyophilised sample was reconstituted in 5 ml of NaCl-TES to give a stock glycan solution. The effect of these glycans on the ability of chicken spermatozoa to undergo the acrosome reaction was assessed by incubating 1.25 x 10^7 spermatozoa ml^{-1} in a 1:10 dilution of the stock sugar solution prepared in NaCl-TES, to a final volume of 1 ml, for 5 min at 30°C. The spermatozoa were assessed for acrosomal status using FITC-PNA labelling of exposed acrosomes as described in Section 2.7.2.3.1.

2.8.3 Enzymatic Cleavage of O- and N- Linked Glycans From Follicular IPVL

All enzymes used for the removal of O- and N- linked glycans were obtained from Oxford Glycosystems, Abingdon. IPVL used in this assay was obtained from follicular oocytes (see Section 2.5.2 for protocol).

Pieces of IPVL of approximately 1.5 cm x 1.0 cm were placed in a 6 well microtitre plate. Removal of N-linked glycans from the IPVL pieces was carried out in the presence of 18 mU ml^{-1} peptide-N-glycosidase F (PNGase F) in 300 µl of 20 mmol l^{-1} sodium phosphate pH 7.5, containing

50 mmol Ethylenediaminetetra-acetic acid disodium salt (EDTA). Removal of O-linked glycans from the IPVL fragments was carried out in the presence of 12 U ml^{-1} Endo-∝-N-acetylgalactosaminidase (O-glycosidase) in 300 µl of 100 mmol l^{-1} sodium citrate-phosphate pH 6.0 containing 100 µg ml^{-1} BSA and 0.02% (w/v) sodium azide. To aid the removal of O-linked glycans 0.7 U ml^{-1} fucosidase, 0.7 U ml^{-1} sialidase and 10 mmol l^{-1} galactonolactone (Kate Gilbert, Oxford Glycosystems, personal communication) were added to the reaction mixture. Control IPVL samples were incubated in the appropriate buffer without the addition of the enzyme. A range of protease inhibitors were added to all wells; these consisted of 1 mmol l^{-1} benzamidine, 10 µg ml^{-1} Trans-epoxysuccinyl-L-leucylamido (4-Guanidin) butane (E-64), 10 µg ml^{-1} Phenylmethylsulfonyl fluoride (PMSF) and 10 µg ml^{-1} pepstatin A. The microtitre plate was incubated at 37°C for 18 h.

2.9 PROTEIN SEPARATION BY SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Protein separation was carried out by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) on 5 - 15% gradient gels (12 cm x 10 cm) according to the method of Laemmli (1970).

2.9.1 Solubilisation of Perivitelline Layer samples

Fragments of approximately 1 cm^2 in area were cut from perivitelline layer samples and added to 100 µl of sample buffer (see Appendix E for composition of electrophoretic reagents). Molecular weights of the proteins were determined using BDH 'Electran' molecular weight markers in the range of 12 - 78 kDa. Samples were boiled for 5 min, centrifuged using a Centurian 8000 microcentrifuge at 13000 g for 3 min and the supernatants were either loaded immediately into sample wells or stored at -20°C until required.

2.9.2 Preparation of 5-15% Resolving Gel

The resolving gel mixture was prepared as shown in Appendix E.4. Gels were poured using a standard gradient maker and a Watson Marlow 101U/R peristaltic pump. The resolving gel solutions were transferred to the appropriate chambers (4 ml per chamber) and allowed to enter the gel plates at a constant rate of approximately 3 ml min^{-1}, until the gel mixture reached 1.7 cm from the top of the glass plate. The gel solution was gently overlaid with water-saturated butanol for approximately 30 min until the gel polymerised.

2.9.3 Preparation of Stacking Gel

The water-saturated butanol was removed from the polymerised resolving gel by several washes with a 1:4 dilution of resolving gel buffer. The stacking gel solution was prepared as shown in Appendix E.5 and poured to the top of the gel plates. A 10 well Teflon comb was inserted into the stacking gel solution and the gel was left to polymerize at room temperature for approximately 30 min.

2.9.4 Protein Separation

Proteins samples were loaded into the wells and separated using a Fisons FEC 185 Dual Mini Vertical Electrophoresis System at a constant current of 15 mA for 1.5 h at 5°C, using a Consort E455 power supply.

2.9.5 Silver Staining of Separated Proteins

After electrophoresis, proteins were visualised according to the method of Blum *et al.* (1987). Solutions containing sodium thiosulphate and silver nitrate were prepared fresh prior to use (see Appendix E.8 for composition of silver staining solutions and protocol).

2.9.6 Protein Band Molecular Weight Determination

Protein band molecular weights were obtained from molecular weight marker (BDH 'Electran') calibration curves using a modification (Sigma Technical bulletin MWS-877L) of the method of Laemmli (1970). Results are mean values from 5 similar gels and identical proteins on subsequent gels were assigned the mean molecular weight values from the original gels.

2.10 ORCINOL-SULPHURIC SUGAR ASSAY

The orcinol-sulphuric sugar assay was used to determine the total sugar concentration in IPVL samples (see White and Kennedy, 1994). A fresh stock solution of 2g l^{-1} orcin monohydrate (orcinol) (Fluka) was prepared in concentrated sulphuric acid (H_2SO_4) and stored at 4°C for up to 1 week.

To duplicate 75 mm tubes, containing either 200 µl of the sample to be analysed or fructose standard solution in the range of 0.00 to 0.1 mg ml^{-1}, 800µl of orcinol reagent was added and mixed well. Tubes were heated to 80°C for 15 mins then rapidly cooled to room temperature. The absorbance of the samples were determined at 420 nm and a standard curve was constructed from which the total sugar content of each sample determined.

2.11 BCA PROTEIN ASSAY

Total protein content of samples was determined using the BCA protein assay (Smith *et al.*, 1985). Stock solutions of 1% (w/v) (BCA)-Na_2, 2% (w/v) Na_2CO_3, 0.16% (w/v) sodium tartrate, 0.4% (w/v) NaOH, 0.95% (w/v) $NaHCO_3$ at pH 11.25 (stock A) and 4% copper sulphate (stock B) were prepared and stored at room temperature until required. Immediately

prior to use, 100 vol of stock A was mixed with 2 vol of stock B to give stock C.

Polycarbonate tubes (75 mm) were set up in duplicate containing a final volume of 50 µl protein sample, or BSA protein standards in the range of 0 mg ml^{-1} to 1 mg ml^{-1}. To each tube, 1 ml of stock C was added and the solution vortexed. The protein solutions were incubated at 37°C for 30 mins and the absorbency read at 562 nm. A standard curve was constructed and used to determine the protein concentration of the samples.

2.12 STATISTICAL ANALYSES

Statistical analysis for the characterisation of the sperm-IPVL assay, utilising IPVL from laid eggs, was carried out by Dr. Harry Staines using MINITAB for windows, version 10, Minitab Inc., USA. All other statistical analysis was carried out using Graph Pad Instat , version 2.04a, Graph Pad software, USA. Means and standard deviations (SD) were calculated before further statistical analysis was carried out. To determine whether two sample means were significantly different, a 2 sample unpaired t-test was used. A one-way ANOVA was carried out to test for significant differences among mean values, when 3 or more samples were being compared in an experiment. When significant differences were detected using the one-way ANOVA, post tests were carried out. Tukey-

Kramer multiple comparisons test was used to compare pairs of data to determine which pair were significantly different, whereas the Dunnetts multiple comparisons test was used to determine which test samples were significantly different from control samples. Two way-ANOVA was used to test for significant differences among mean values when testing the effect of one factor (e.g. the number of points of hydrolysis), while controlling for different sources of experimental material (i.e. different sources of IPVL). An analysis of covariance (ANCOVA) was used to assess the linear relationship between sperm concentration and the mean number of points of hydrolysis, while controlling for differences in the source of IPVL. The Kruskal-Wallis test was used as an alternative to ANOVA for particularly skewed data.

CHAPTER 3 RESULTS

3. RESULTS

3.1 SEPARATION OF INNER AND OUTER PERIVITELLINE LAYERS FROM LAID EGGS

Incubation of isolated yolks from chicken, turkey, duck, peahen and pheasant laid eggs in 0.01mol l^{-1} HCl for 1 h at 37°C (see Section 2.5.4 for protocol) caused an increase in yolk volume, which resulted in separation of the inner and outer perivitelline layers as described by Kido and Doi (1988). Figure 8A shows isolated yolks from turkey and chicken eggs immersed in the acid before incubation at 37°C for 1 h; Figure 8B shows the increase in yolk volume after 1 h incubation at 37°C. When the burst and cleaned perivitelline layers were laid out in a petri dish, the inner layer could easily be distinguished from the outer layer as a thinner, more transparent structure (Figure 9). Kido and Doi (1988) found that chicken eggs stored at 4°C for more than 24 h separated poorly. In contrast, in the present work, it was found that good separation of the layers could be obtained with laid eggs, stored at room temperature for up to 3 days, with the exception of turkey eggs which failed to separate after 24 h storage.

Separation of the inner and outer perivitelline layers from zebra finch eggs was achieved by simply pulling apart the two layers under a

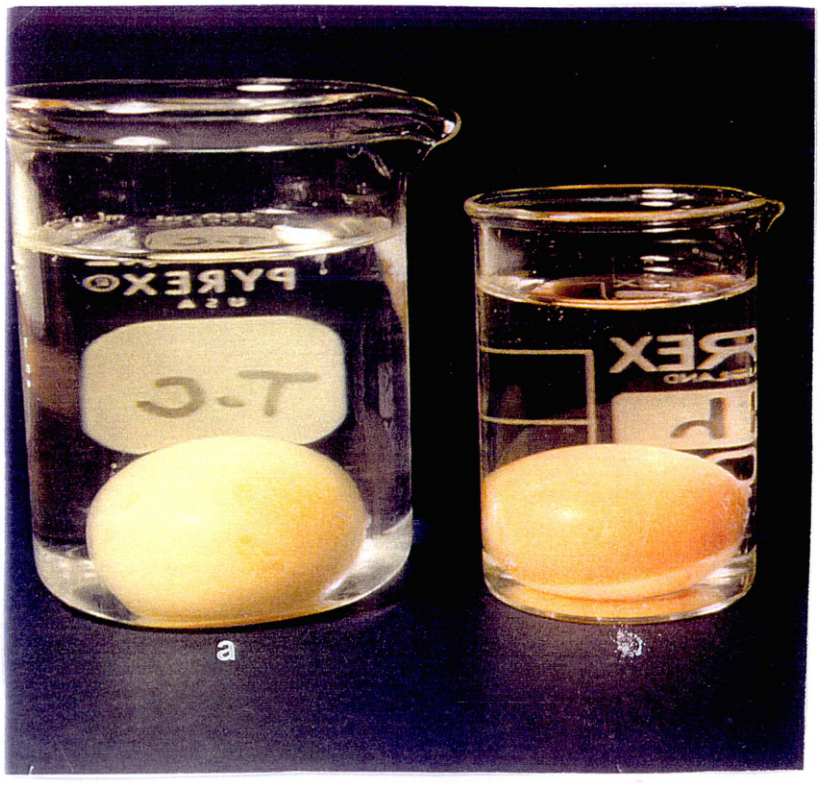


Figure 8A. Isolated yolks from laid turkey (a) and chicken (b) eggs immersed in 0.01 mol HCl l^{-1} prior to incubation at 37°C .

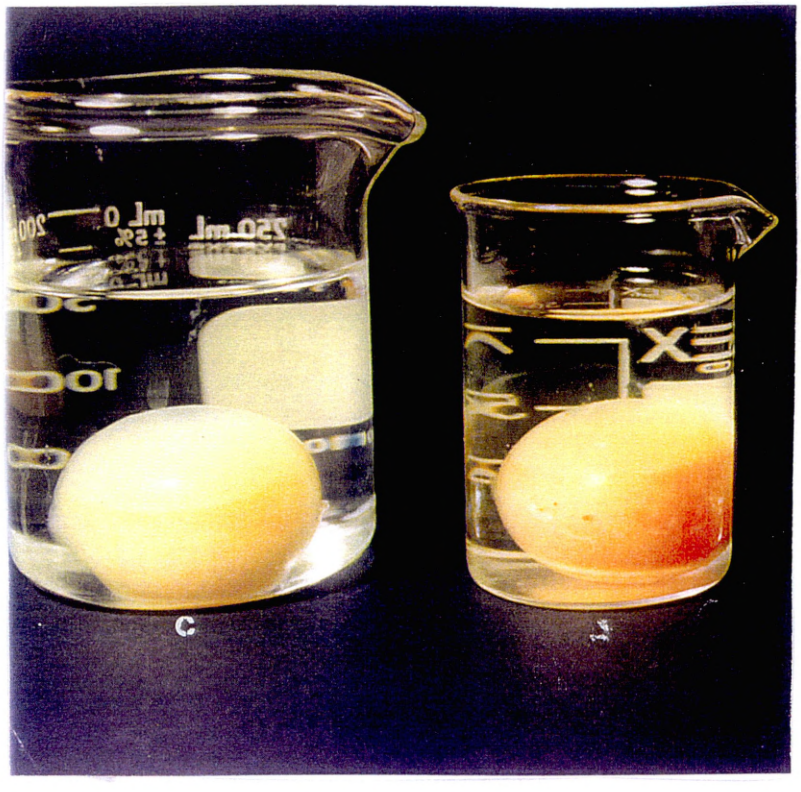


Figure 8B. Increase in yolk volume after incubation of isolated turkey (c), and chicken (d) yolks in 0.01mol HCl l^{-1} for 1 h at 37°C.

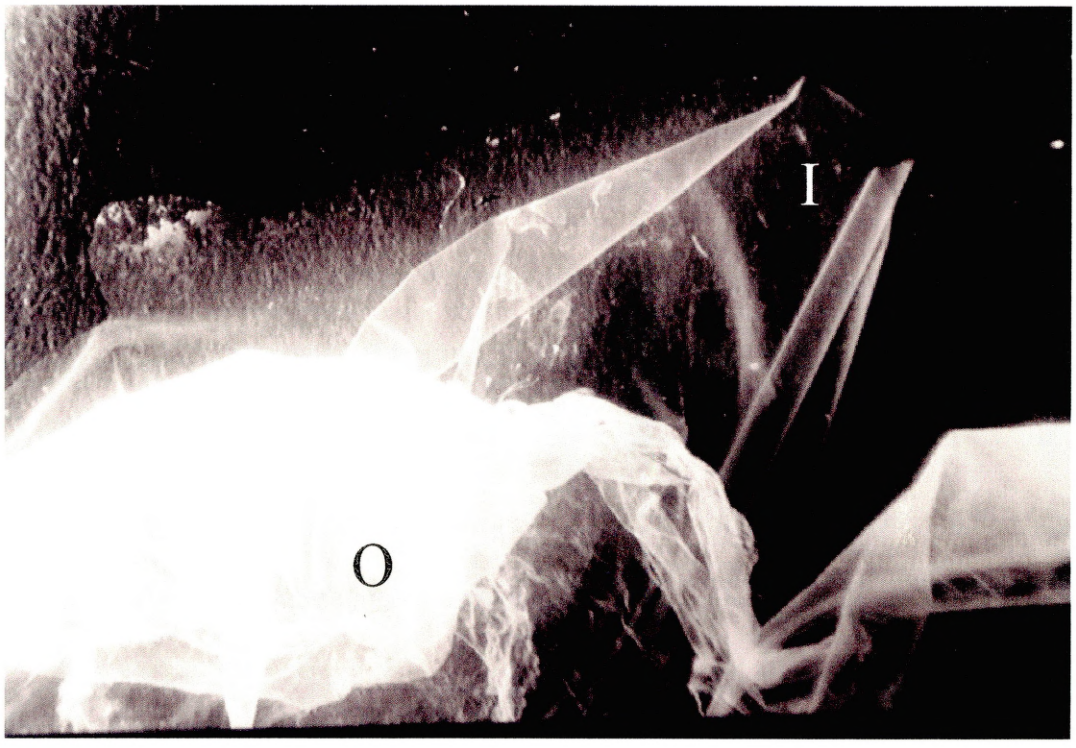


Figure 9. Separation of inner and outer perivitelline layers from a laid chicken egg. The inner layer (I) can be distinguished from the outer (O) layer, as a thinner, more transparent structure.

3.2 ELECTROPHORETIC PROFILES OF CHICKEN PERIVITELLINE LAYERS

The protein profiles obtained from both ovulated and follicular IPVL appeared to be identical with 2 major bands at 78 kDa and 39 kDa (Figure 10 d, e). A 78 kDa band was also present in the inner and whole perivitelline layer from laid eggs, but the 39 kDa protein was absent and replaced by a 35 kDa band (Figure 10 a, c). The intensity of the bands obtained for the follicular IPVL, was consistently less than in the ovulated and laid IPVL samples of the same size. Neither of these bands were present in the outer perivitelline layer separated from laid eggs (Figure 10 b).

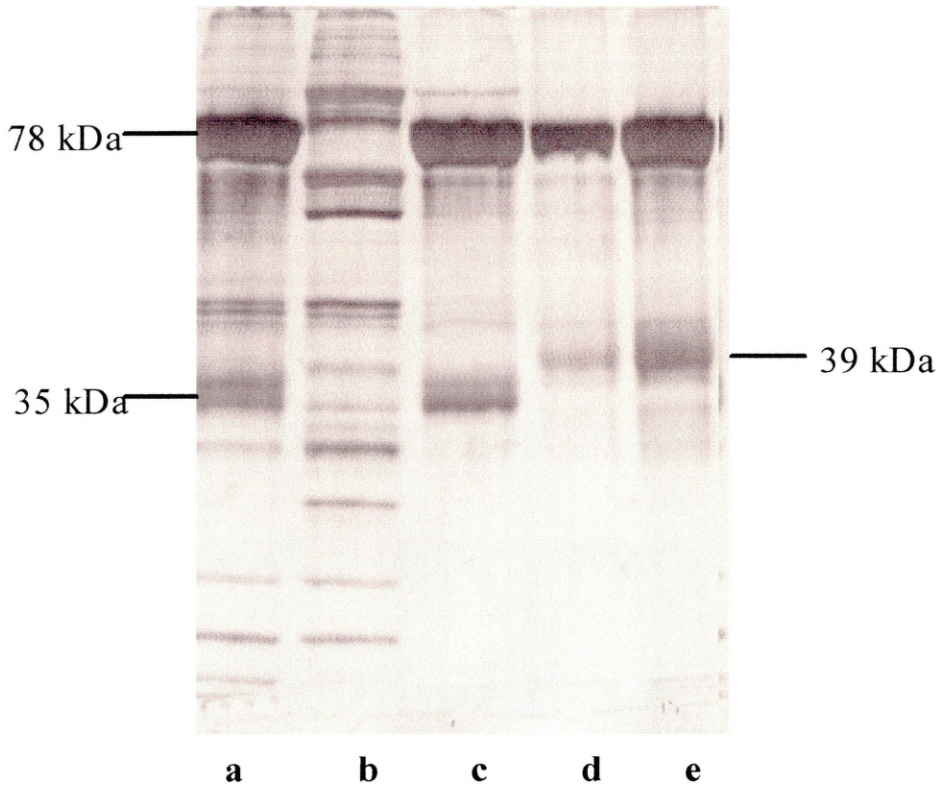


Figure 10. SDS-PAGE protein profiles of the whole (a), outer (b) and inner (c) perivitelline layers from a laid chicken egg with inner layer from follicular (d) and ovulated (e) ova. All samples were taken from the same bird. In all cases aliquots of 3.5 µl of solubilised proteins, taken from 1 cm^2 sheets of IPVL, were loaded onto the gel. Protein bands were visualised by silver staining after the method of Blum *et al.* 1987.

3.3 CHARACTERISATION OF THE SPERM-EGG INTERACTION ASSAY USING IPVL FROM LAID CHICKEN EGGS

The suitability of IPVL separated from laid chicken eggs was investigated as a readily-available source of material for studying sperm-egg interaction *in vitro.* Although Kido and Doi (1988) demonstrated that the IPVL could be separated from the OPVL of laid eggs by acid hydrolysis and that it retained some structural integrity, no details were available on its functional status. Therefore, in this work the response of IPVL separated from laid chicken eggs was investigated as a substrate for hydrolysis by spermatozoa *in vitro* and the system was further characterised as a quantitative assay, in which the end-point was measured as the number of points of hydrolysis produced in the IPVL. For each experimental unit, 3 fragments were taken from each IPVL and 3 fields of view examined for each fragment in the *in vitro* sperm-IPVL assay as described in Section 2.6.3 unless otherwise stated.

3.3.1 Comparison of the Response of IPVL from Ovulated, Follicular and Laid Eggs to Hydrolysis by Chicken Spermatozoa

IPVL from ovulated, follicular and laid eggs, from the same bird, showed a similar ability to be hydrolysed by the same pooled sample of spermatozoa. The mean frequency of points of hydrolysis from 3 experiments, each using IPVL from different hens, are shown in Figure 11. A nested two-way ANOVA showed no significant difference ($P > 0.05$) in the mean number of points of hydrolysis among the types of IPVL for individual hens, but significant differences ($P < 0.0005$) were observed in samples taken from different hens.

3.3.2 Effect of Inter- and Intra- Egg Variation in the Response of IPVL to Hydrolysis by Spermatozoa

Variability in the response of IPVL to hydrolysis by spermatozoa was investigated by determining the ability of pieces of IPVL from the same, and from different laid eggs to be hydrolysed by the same pooled sample of semen.

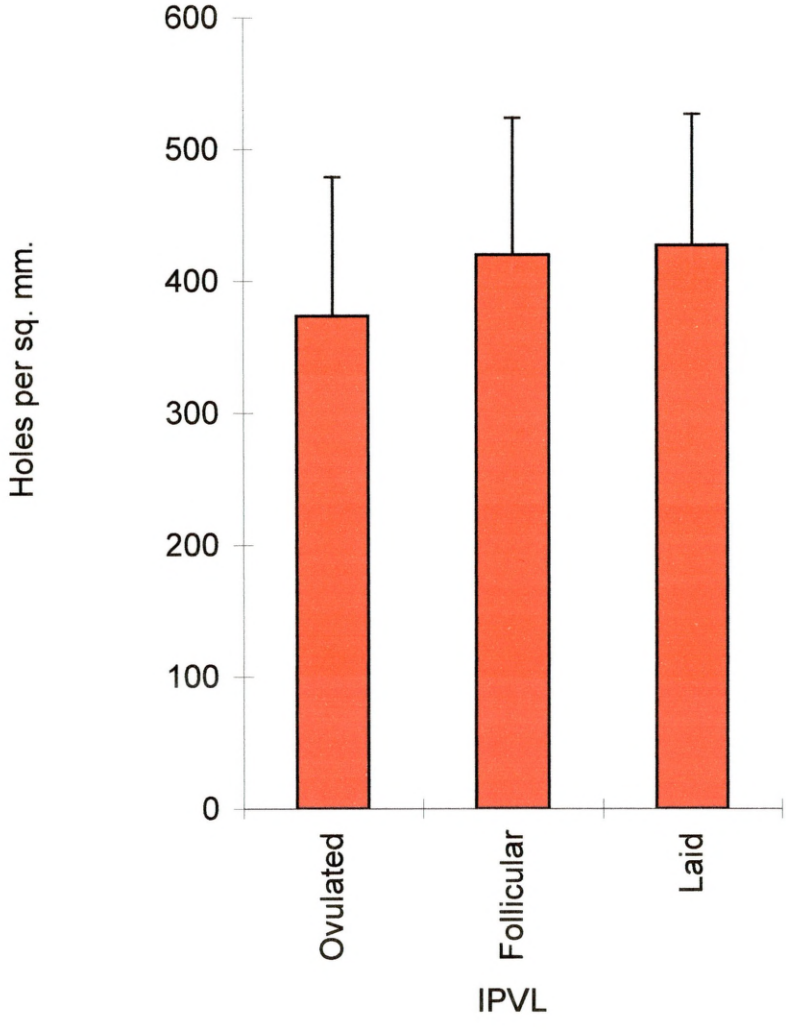


Figure 11. Frequency of points of hydrolysis produced in IPVL from ovulated, follicular and laid eggs from the same bird incubated with 1.25 $x10^7$ spermatozoa ml^{-1}. Results are mean ± SD from 3 experiments.

The mean frequency of points of hydrolysis from experiments in which IPVL separated from 4 laid eggs, each from different hens, and incubated with the same pooled sample of spermatozoa are shown in Figure 12. A nested two-way ANOVA showed significant differences in the mean number of points of hydrolysis between IPVL pieces from different eggs, taken from different females ($P < 0.0005$). However, no significant difference was found among the mean number of points of hydrolysis in IPVL pieces taken from the same egg (nested two-way ANOVA; $P > 0.05$).

3.3.3 Effect of Spermatozoa Samples from Different Males on Hydrolysis of the IPVL

Sperm samples from individual male birds were found to have a different and characteristic ability to hydrolyse pieces of IPVL in the *in vitro* sperm-IPVL assay. In 3 experiments, carried out on consecutive weeks, semen samples from 8 different males, each containing 1.25×10^7 spermatozoa ml^{-1}, were incubated with a piece of IPVL from the same laid egg and 3 fields of view examined. The Kruskal-Wallis test showed significant differences ($P < 0.005$) in the resultant median frequencies of points of hydrolysis produced in the IPVL by the different male birds (Figure 13).

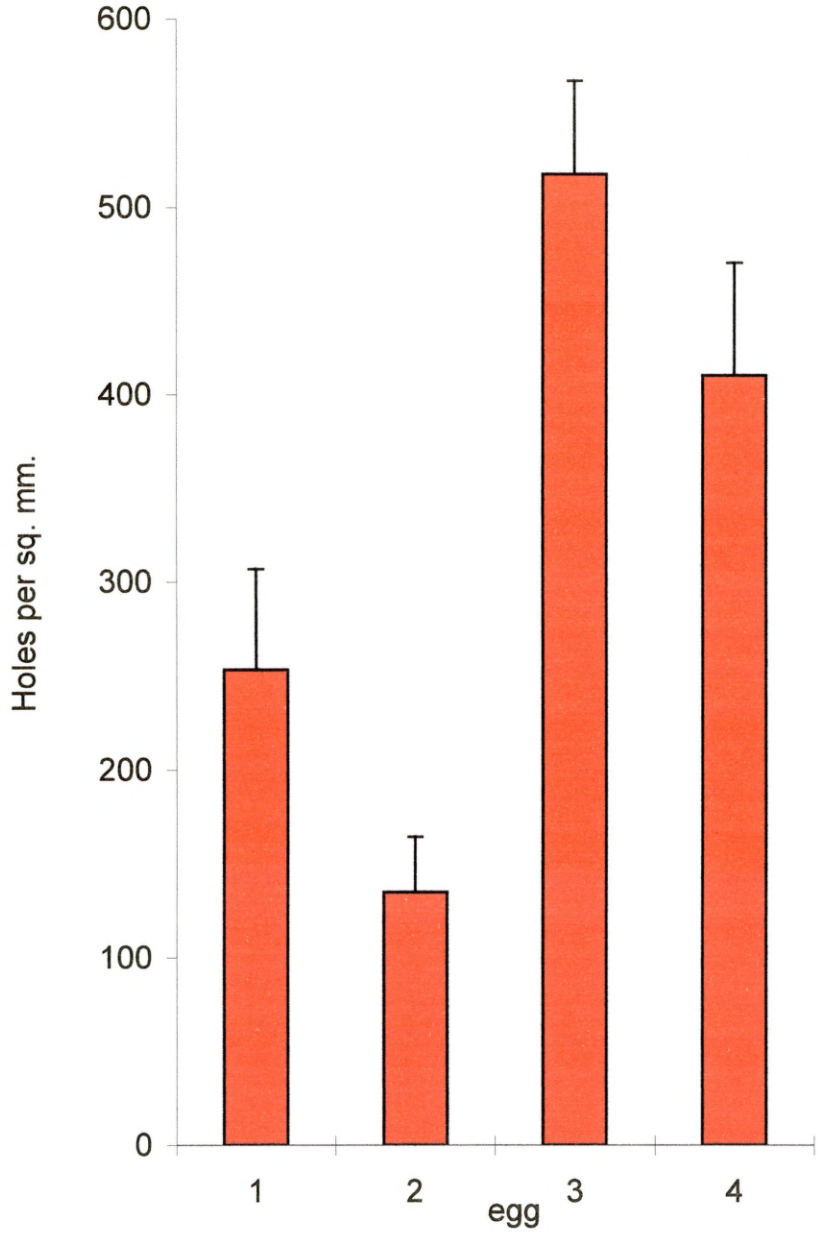


Figure 12. Frequency of points of hydrolysis produced by 1.25 x 10^7 spermatozoa ml^{-1} in pieces of IPVL separated from laid chicken eggs. Results are mean ± SD from 3 experiments in which IPVL from 4 eggs, each from different birds, were incubated with the same pooled sample of semen.

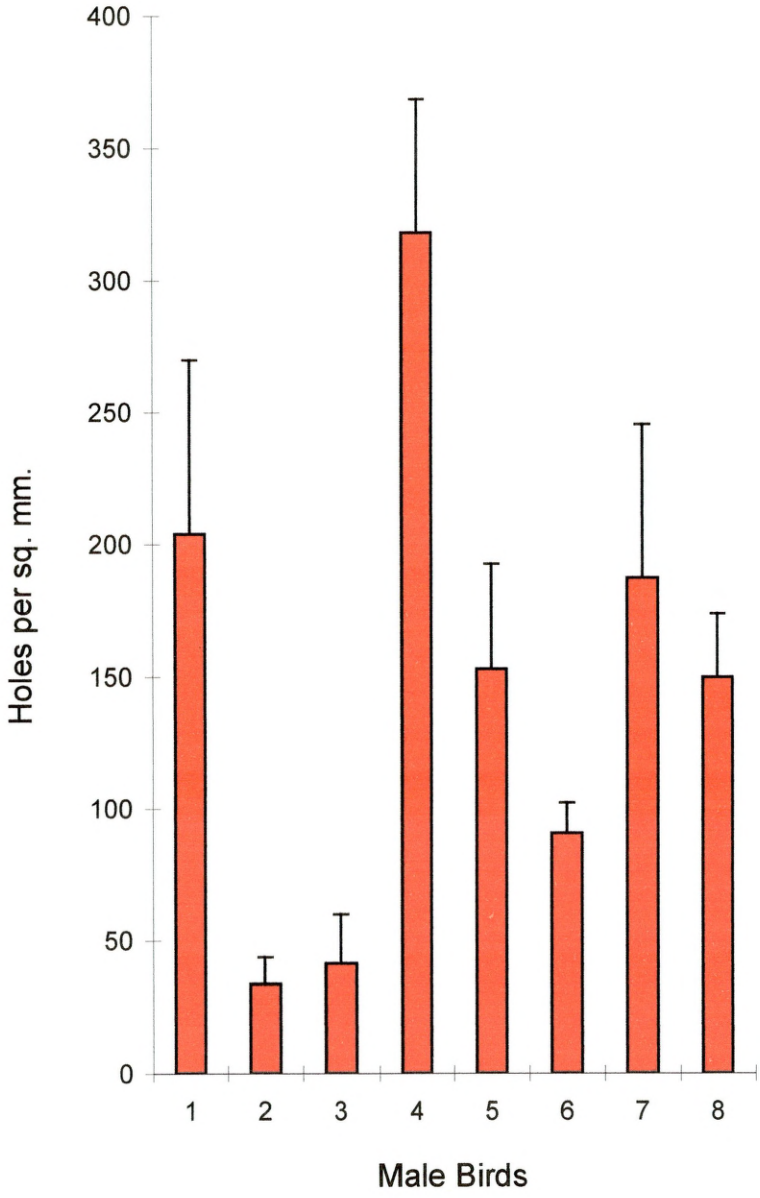


Figure 13. Frequency of points of hydrolysis produced in pieces of IPVL, from the same laid egg, incubated with spermatozoa samples (1.25 x 10^7 spermatozoa ml^{-1}) from 8 different male birds. Results are mean ± SD from 3 experiments

3.3.4 Relationship between Sperm Concentration and Frequency of Points of Hydrolysis in IPVL from Laid Eggs

The frequency of points of hydrolysis produced in IPVL from laid eggs, was found to increase linearly with increasing sperm concentration used in the assay (Figure 14). An analysis of covariance (ANCOVA) showed that there was a significant linear relationship between spermatozoa concentration and the number of points of hydrolysis made in the IPVL ($P < 0.01$) after controlling for different semen and IPVL samples ($P < 0.01$). However, at sperm concentrations greater than 62.5×10^6 spermatozoa ml^{-1}, counts became inaccurate as holes started to merge, eventually resulting in disintegration of the IPVL. At concentrations below 6.25×10^6 spermatozoa ml^{-1} the number of points of hydrolysis was more than predicted from extrapolation of the results. Photomicrographs of IPVL samples taken from the same egg, after incubation with increasing concentrations of spermatozoa from the same semen sample, are shown in Figure 15.

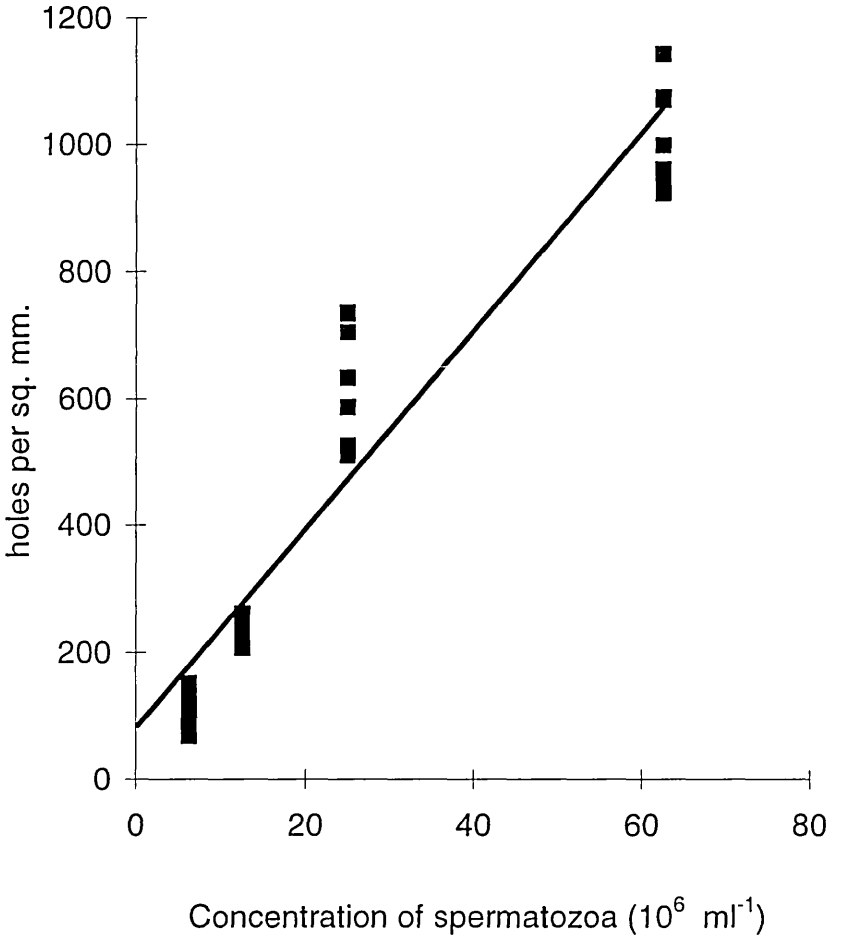


Figure 14. Relationship between sperm concentration and the number of points of hydrolysis in the IPVL from laid chicken eggs *in vitro*. In 3 experiments, using different spermatozoa and IPVL samples, the relationship was found to be holes mm^{-2} in the inner perivitelline layer = 15.65 + 80.94 x 10^6 spermatozoa ml^{-1}; r = 0.96.

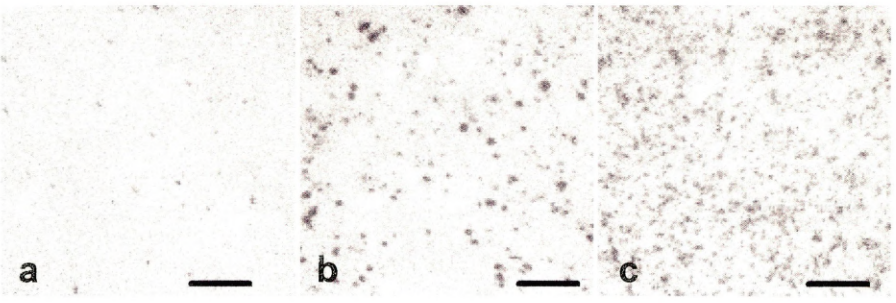


Figure 15. Points of hydrolysis present as dark 'holes' in the IPVL after incubation with increasing concentrations of spermatozoa (a: 5 $\times 10^6$ sperm ml^{-1}; b: 25×10^6 sperm ml^{-1}; c: 50×10^6 sperm ml^{-1}). Scale bar represents 150 µm.

3.3.5 Determination of the Optimum Time of Hydrolysis of the IPVL by Spermatozoa

Hydrolysis of the IPVL by spermatozoa was first identified after a 2.5 min incubation period, at which time the holes were between 5 µm and 10 µm in diameter. After incubation for 5 min, the number of holes in the IPVL did not increase significantly, but the diameter of the holes increased from 10 µm to 20 µm. Beyond 10 min incubation the average size of the IPVL-holes increased to between 30 - 50 µm diameter and the holes started to merge, making counts inaccurate, eventually resulting in disintegration of the IPVL.

In 3 experiments using IPVL from the same laid egg, a one-way ANOVA showed significant differences in the number of points of hydrolysis in IPVL samples among the different sampling times ($P < 0.001$). However, further analysis using Tukey-Kramer multiple comparisons test, showed there was no significant difference between the mean frequency of points of hydrolysis in the IPVL at sampling times of 2.5 and 5 min ($P > 0.05$), but the mean number of points of hydrolysis at 10 min were significantly lower ($P < 0.001$) than those of the earlier time points (Figure 16).

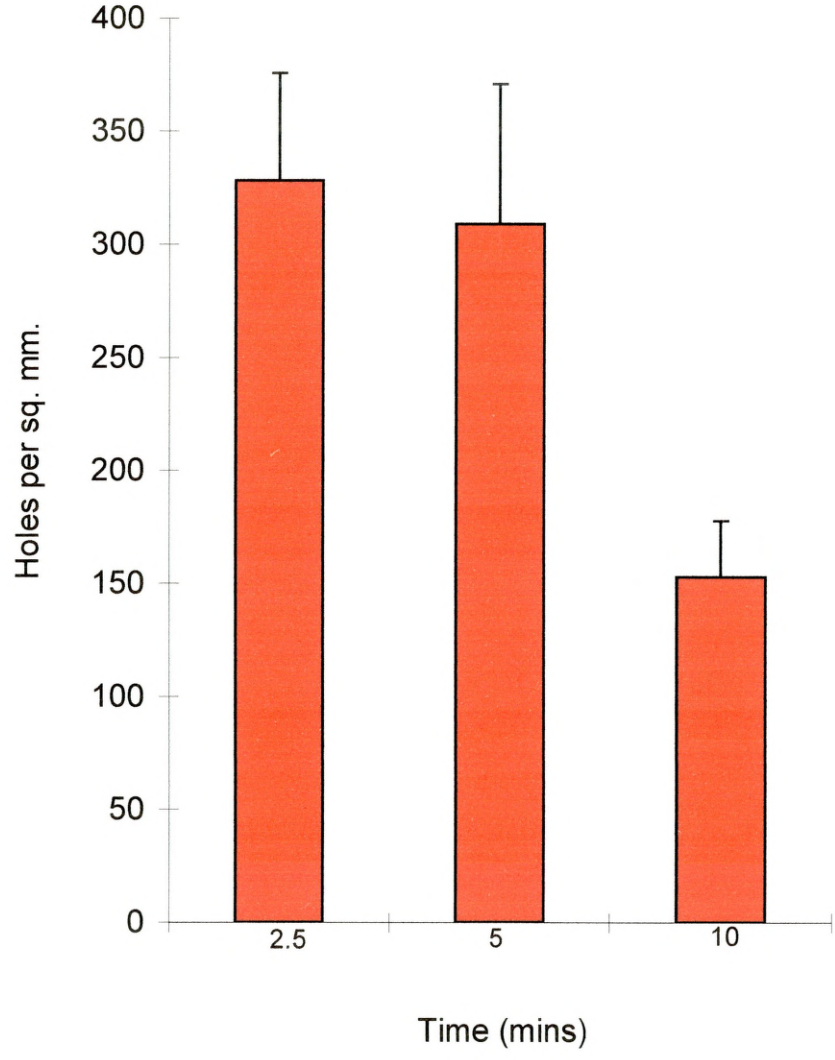


Figure 16. Frequency of points of hydrolysis produced in the IPVL from laid chicken eggs following incubation with 1.25×10^7 sperm ml^{-1} for 2.5, 5 and 10 mins. Results are mean ± SD of 3 experiments.

3.4 APPLICATION OF THE *IN VITRO* SPERM-IPVL INTERACTION ASSAY IN ASSESSING SPERM 'QUALITY' AND FERTILIZING ABILITY OF SEMEN

Current *in vitro* methods for assessing sperm 'quality' rely on assessment of single parameters such as sperm morphology, ATP, motility, (Wishart and Palmer, 1986) or metabolic activity (Chaudhuri and Wishart, 1988). Although the results of these tests have been shown to be highly correlated with the fertilizing ability of fresh semen, and have demonstrated significant differences in the fertility of individual male birds, (see Wishart, 1995) they have not been widely applied. Furthermore, current sperm quality assays, although adequate for fresh semen, tend to grossly overestimate the fertilizing ability of semen that has been stored. It has been presumed that under storage conditions, particularly at 5°C, spermatozoa are damaged in some subtle way which is not detected by the currently-available sperm quality tests (see Wishart, 1995). The sperm-IPVL assay however, requires that sperm are able to perform a series of activities including - motility, binding to the IPVL, induction of the AR and hydrolysis of the IPVL. Therefore, the *in vitro* sperm-IPVL assay utilising IPVL separated from laid eggs was investigated as a method of predicting the fertilizing ability of both fresh and stored semen and was compared with standard methods of assessing sperm quality.

3.4.1 Evaluation of the Fertilizing Ability of Fresh Ejaculates of Semen from Individual Male Birds

3.4.1.1 Comparison of sperm ATP content from individual male fowl with the number of points of hydrolysis produced in the *in vitro* sperm-IPVL assay

Different male birds were found to differ significantly in their cellular ATP content (Kruskal-Wallis; $P < 0.007$; n = 24) as shown before (Wishart and Palmer, 1986; Chaudhuri *et al.*, 1988). The results of the sperm ATP assay were found to correlate linearly with the individual results of the *in vitro* sperm-IPVL assay for each male bird (see Section 3.3.3 for results of the *in vitro* sperm-IPVL assay) in 3 experiments carried out on consecutive weeks (Figure 17).

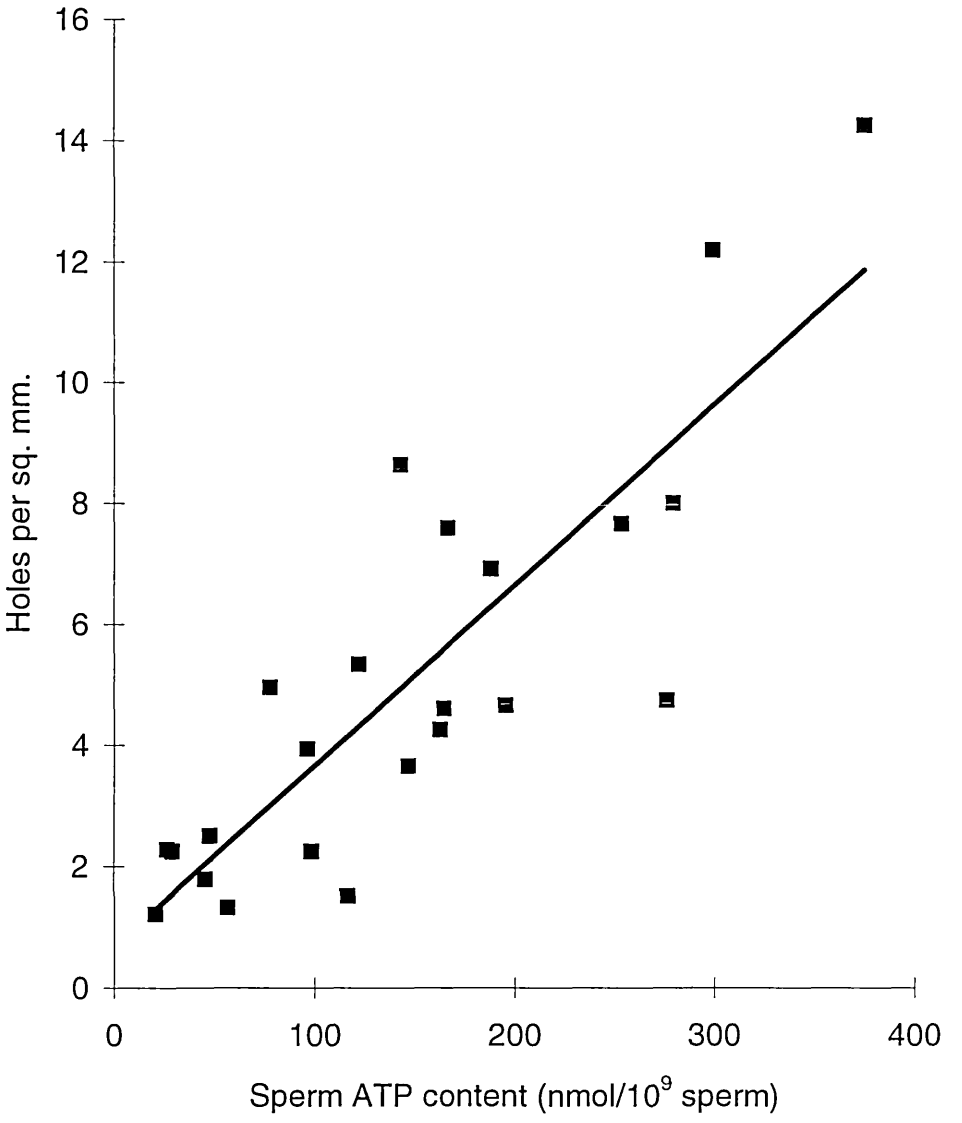


Figure 17. Relationship between IPVL-holes formed *in vitro* and sperm ATP concentration. The relationship is holes mm^{-2} = 24 nmol ATP/10^9 sperm + 24; r=0.85; n=24.

3.4.1.2 Comparison of the number of IPVL-holes formed in the *in vitro* sperm-IPVL assay with the number of IPVL-holes formed *in vivo* in fertilized laid eggs and the percentage of fertile eggs laid

The mean frequency of points of hydrolysis in the *in vitro* sperm-IPVL assay, using semen from birds 1, 2, 3, 4, 6 and 8 (see Section 3.3.3 for results of *in vitro* sperm-IPVL assay for each individual male bird), correlated linearly with the mean number of holes produced in the IPVL *in vivo*, at sites away from the animal pole, in eggs laid by hens inseminated with semen (see Section 2.6.1 for method) from the same male birds (Figure 18a). The mean IPVL-holes formed in the *in vitro* assay also correlated with the logarithm of the proportion of fertile eggs laid by the same birds (Figure 18b).

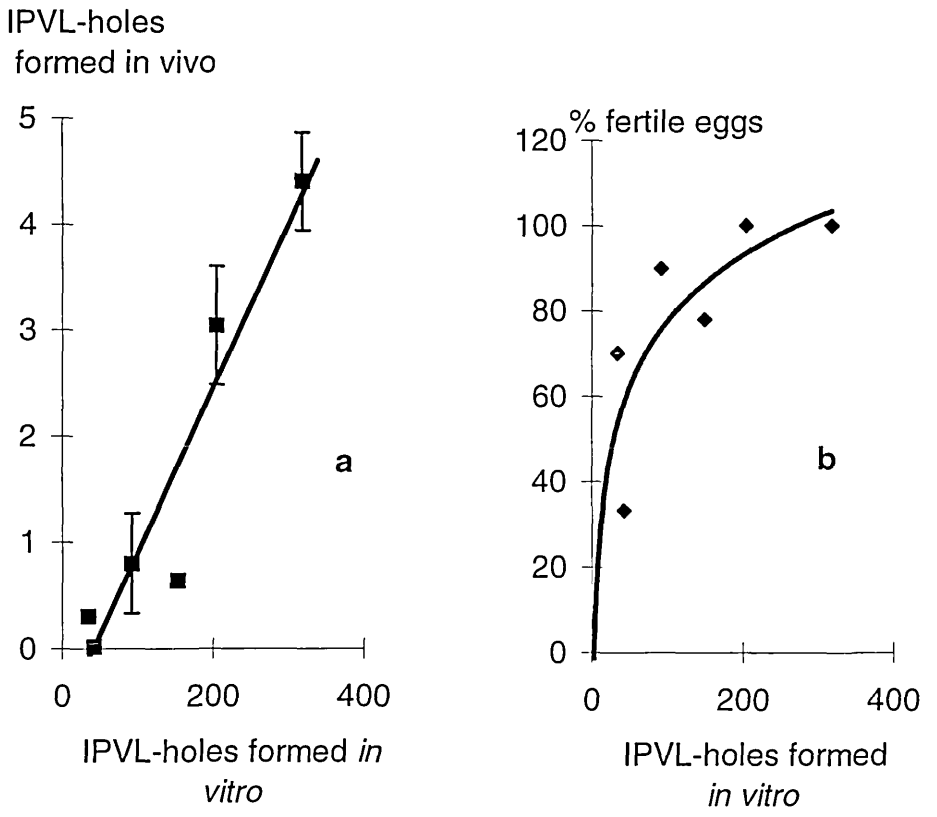


Figure 18. The relationship between IPVL-holes formed *in vitro* with (a) IPVL holes from laid eggs fertilized *in vivo* by inseminated sperm and (b) the proportion of fertile eggs in the same eggs. Groups of 5 hens were inseminated 3 times with 60 x 10^6 sperm from each of 6 individual male fowl; eggs were collected on day 6. The mean (± SD) of IPVL-holes formed *in vivo* (a) and the percentage of fertile eggs (b) laid are plotted against the mean (± SD) of IPVL-holes formed *in vitro* by spermatozoa from each of the males. Equations are for (a): y = 0.015x -0.62; r=0.79; and for (b) y = 22.32 logx - 25.15; r^2 =0.61.

3.4.2 Application of the *In Vitro* Sperm-IPVL Assay in Assessing the Fertilizing Ability of Stored Semen

3.4.2.1 Effect of aerobic and anaerobic storage of chicken semen at 5°C on sperm activity

The ability of chicken spermatozoa to exclude eosin, or metabolise INT-tetrazolium did not differ significantly between fresh semen samples and those stored under aerobic or anaerobic conditions for 24 h at 5°C (one-way ANOVA; each $P > 0.1$). However, a significant difference was seen in the ability of fresh and stored semen samples to hydrolyse the IPVL in the *in vitro* sperm-IPVL assay (one-way ANOVA; $P < 0.0001$) (Figure 19). Further analysis using Tukey-Kramer multiple comparisons test showed a significant reduction in the ability of stored semen to hydrolyse the layer compared with fresh semen samples (each $P < 0.001$). Furthermore this was the only test to differentiate between the 2 methods of storage ($P < 0.01$), with only approximately 5% of anaerobically stored samples retaining the ability to hydrolyse the layer, compared with 52% for spermatozoa stored aerobically.

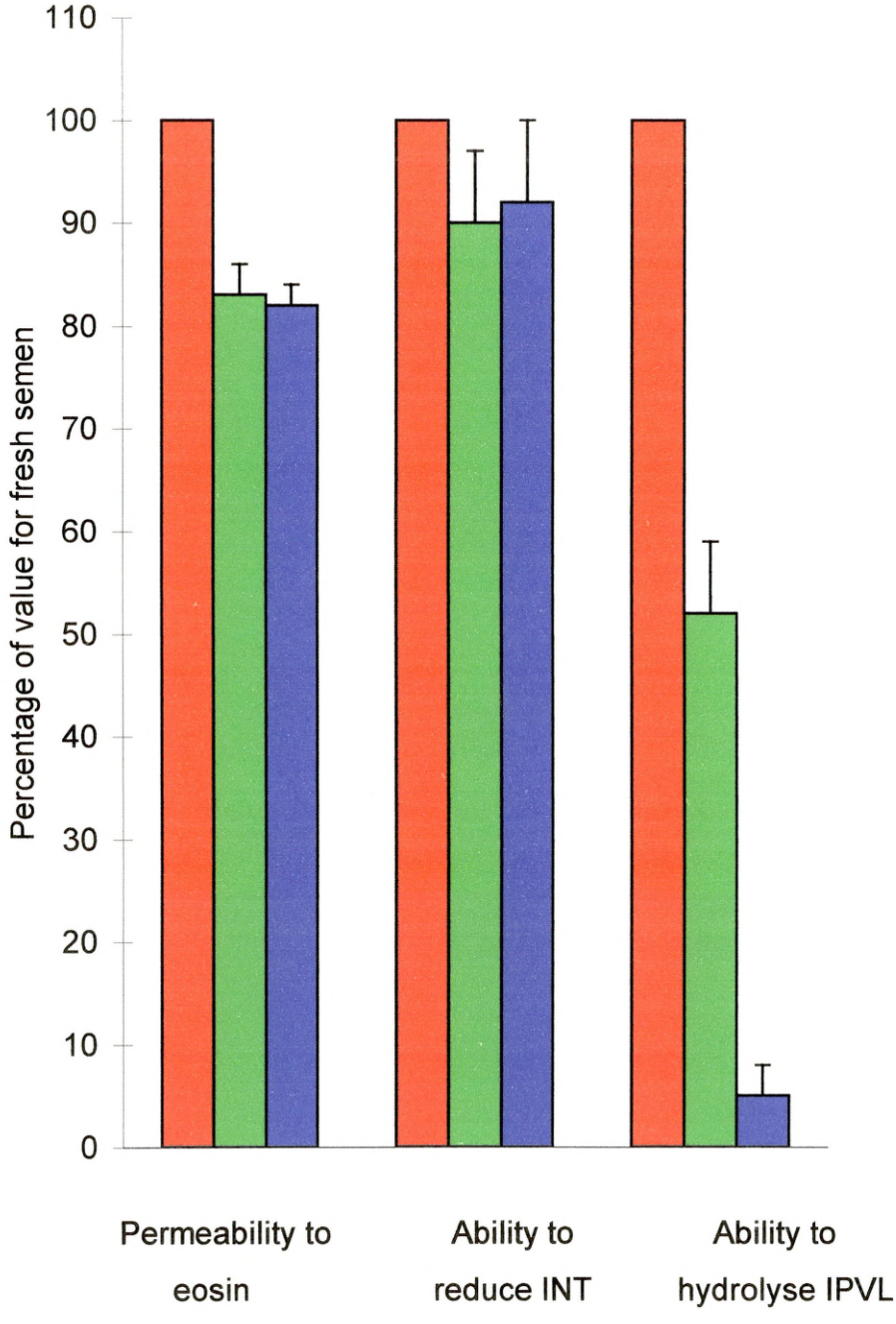


Figure 19. Effect of chicken semen storage at 5°C for 24 h on sperm eosin permeability, capacity of spermatozoa to reduce the tetrazolium salt INT and their ability to produce points of hydrolysis in the IPVL of chicken eggs. Results (means of 3 experiments) are given as percentages of values of fresh semen (■). Samples were stored aerobically by shaking (■), or anaerobically (■) in sealed tubes.

3.4.2.2 Effect of aerobic and anaerobic storage of turkey semen at 5°C on sperm activity

Following storage for 24 h at 5°C, under aerobic and anaerobic conditions, turkey spermatozoa showed a significant reduction in the ability to exclude eosin compared with fresh semen samples (one-way ANOVA; $P < 0.01$) (Figure 20). Further analysis using Tukey-Kramer multiple comparisons test, showed a significant difference in eosin-permeability between fresh semen samples and those stored under both aerobic and anaerobic conditions ($P < 0.01$ and 0.05 respectively), but no significant difference between the 2 methods of storage ($P > 0.05$).

The ability of turkey spermatozoa to metabolise the tetrazolium salt -INT was also significantly different between fresh and stored semen samples (one-way ANOVA; $P < 0.0005$). Stored samples showed a reduced ability to metabolise INT compared with fresh semen samples for the same males (Tukey-Kramer multiple comparisons test; $P < 0.05$ for aerobically stored spermatozoa; $P < 0.001$ for anaerobically stored spermatozoa) and a significant difference was also seen in the ability of spermatozoa to reduce INT between the two methods of storage ($P < 0.05$).

The greatest effect on sperm activity following 24 h storage at 5°C, was observed in the ability of turkey spermatozoa to hydrolyse the IPVL in the *in vitro* assay (one-way ANOVA; $P < 0.0001$). Both aerobically and anaerobically stored spermatozoa showed a significant decrease in ability to hydrolyse the

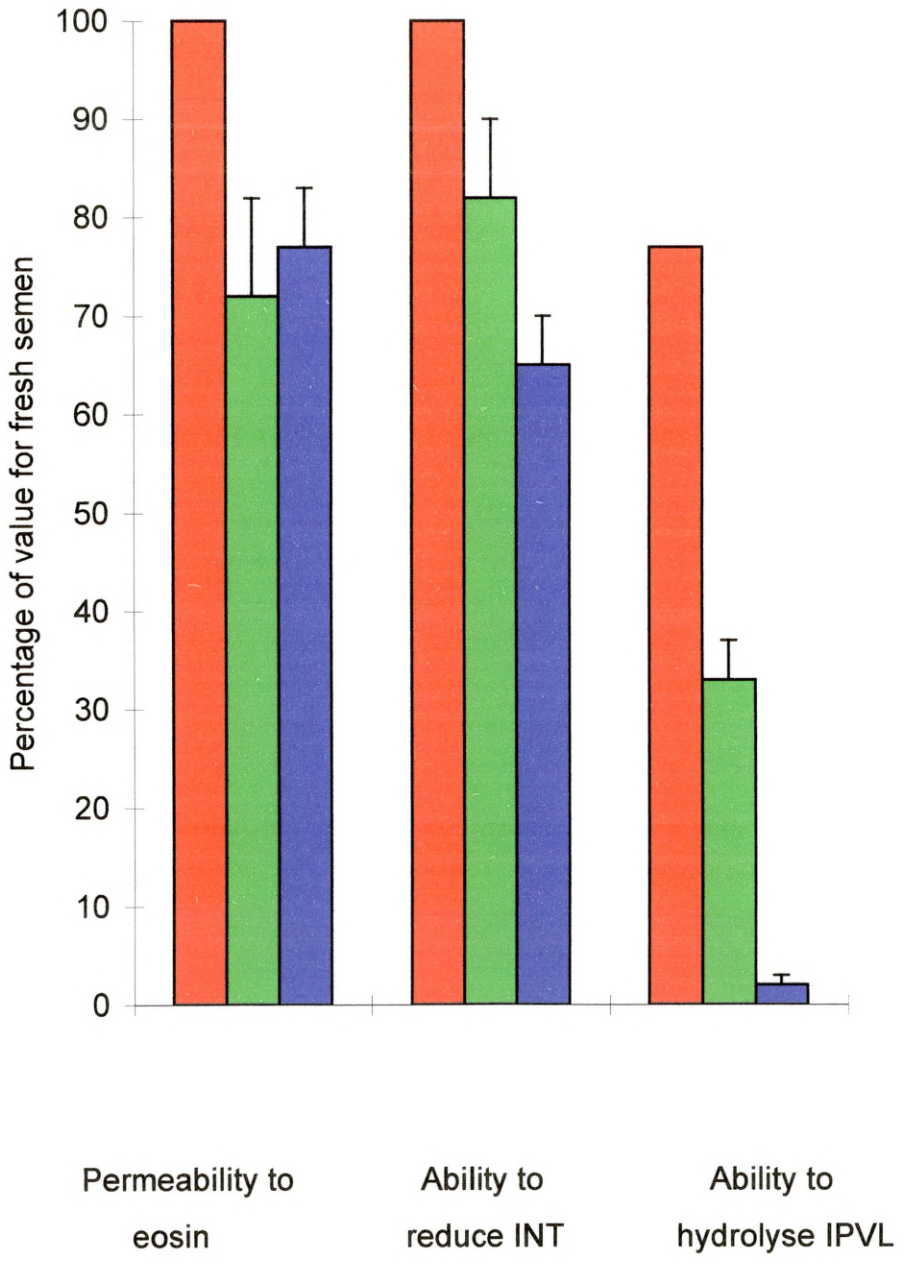


Figure 20. Effect of storage on turkey semen activity at 5°C for 24 h on sperm eosin permeability, capacity of spermatozoa to reduce the tetrazolium salt INT and their ability to produce points of hydrolysis in the IPVL of laid turkey eggs. Results (means of 3 experiments) are given as percentage of values recorded for fresh semen (■). Samples were stored aerobically by shaking (■), or anaerobically (■) in sealed tubes.

IPVL when compared to fresh semen samples. However, a significant difference was also seen between the 2 methods of storage. Approximately 32% of aerobically stored turkey spermatozoa retained the ability to bind to and hydrolyse the IPVL after 24 h, whereas this ability was virtually lost in samples stored anaerobically under the same conditions (Tukey-Kramer multiple comparisons test; each $P < 0.01$).

3.5 SPECIES SPECIFICITY OF SPERM-IPVL INTERACTION

3.5.1 Electrophoretic Profiles of IPVL Separated from Laid Eggs of Different Avian Species

The electrophoretic profiles of IPVL from chicken (Figure 21 a), turkey (Figure 21 b), peahen (Figure 21 c), duck (Figure 21 d) and zebra finch (Figure 21 e) (see 2.9 for protocol) showed the presence of a major band at 78 kDa, as found before for chicken IPVL (see Section 3.2). A 35 kDa band was present in chicken IPVL (Figure 21 a) as shown previously (see Section 3.2), but was absent in all other species assessed. This band was replaced by a 39 kDa band in turkey (Figure 21 b) and peahen IPVL (Figure 21 c) and by a 40 kDa band in duck (Figure 21 d) and zebra finch IPVL (Figure 21 e).

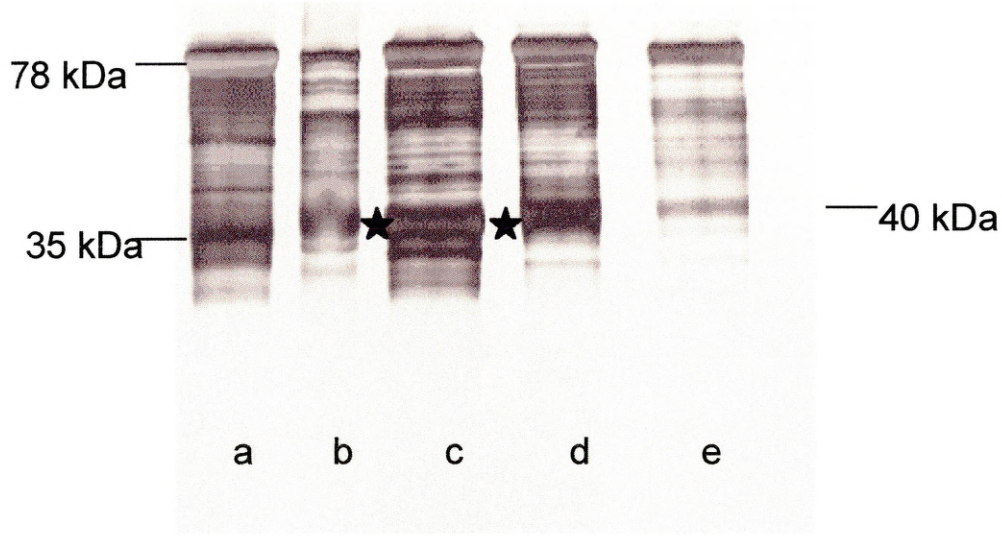


Figure 21. SDS-PAGE protein profiles of IPVL from chicken (a), turkey (b), peahen (c), duck (d) and zebra finch (e) laid eggs. Two major bands can be seen. A 78 kDa band was present in all species. A second major protein band was present at 35 kDa in chicken IPVL. The second major band was at 39 kDa band in turkey and peahen IPVL (identified by a ★ at the right side of each lane) and 40 kDa in duck and zebra finch IPVL. In all cases aliquots of 3.5 µl of solubilised proteins, taken from 1 cm^2 pieces of IPVL, were loaded onto the gel. Protein bands were visualised by silver staining after the method of Blum *et al.* 1987.

3.5.2 Interaction of Chicken Spermatozoa with IPVL Separated from Laid Eggs of Different Avian Species

Chicken spermatozoa were found to hydrolyse the IPVL separated from laid eggs in all avian species tested. In each experiment 1.25×10^7 spermatozoa ml^{-1} were incubated with 3 pieces of IPVL from the same laid egg and 3 fields of view analysed (see Section 2.6.3 for protocol).

Significant differences were seen in the ability of chicken spermatozoa to hydrolyse the IPVL separated from turkey, pheasant, peahen, duck and zebra finch laid eggs (one way ANOVA; $P < 0.0001$) (see Table 1). Chicken spermatozoa produced more points of hydrolysis in turkey IPVL than in homologous IPVL (Dunnetts multiple comparisons test; $P < 0.05$). Chicken spermatozoa were also able to hydrolyse pheasant IPVL with more than 90% efficiency (Dunnetts multiple comparisons test; $P < 0.05$) and peahen IPVL with approximately 65% efficiency ($P < 0.01$). This ability was greatly reduced in IPVL from duck and zebra finch (Dunnetts multiple comparisons test; $P < 0.01$ in all cases).

Source of IPVL	Order	% IPVL-holes produced by chicken spermatozoa
Chicken	*Galliformes*	100.00 ± 0.0%
Turkey	*Galliformes*	111.71 ± 10.0%
Pheasant	*Galliformes*	91.90 ± 15.3%
Peahen	*Galliformes*	64.34 ± 12.2%
Zebra finch	*Passeriformes*	26.03 ± 5.6%
Duck	*Anseriformes*	18.6 ± 6.6%

Table 1. Interaction of chicken spermatozoa with IPVL separated from laid eggs of various avian species *in vitro*. Results (mean ± SD of 3 experiments) are expressed as percentage values of chicken spermatozoa binding to homologous IPVL.

3.5.3 Interaction of Spermatozoa from Mammalian Species with IPVL Separated from Laid Chicken Eggs

Pieces of IPVL from laid chicken eggs (see Section 2.5.4 for protocol) were incubated with either capacitated or uncapacitated mouse, rat (cauda epididymis) or cryopreserved bull spermatozoa (ejaculated) (see Section 2.3.6 for capacitation protocols) in the *in vitro* sperm-IPVL assay (see Section 2.6.3 for protocol) for either 5 or 30 min. Spermatozoa from these species did not bind to, or hydrolyse the IPVL and no difference was seen between capacitated and uncapacitated spermatozoa.

3.6 NATURE OF THE PREFERENTIAL HYDROLYSIS OF THE IPVL OVERLYING THE GERMINAL DISC

3.6.1 Assessment of the Preferential Hydrolysis of the IPVL Overlying the GD Following *In Vitro* Fertilization

Incubation of chicken spermatozoa with intact ovulated ova *in vitro* (see Section 2.6.2 for method) resulted in an increased number of points of hydrolysis in the IPVL overlying the GD region, compared with the IPVL away from this region (non-GD IPVL). Following incubation of the intact ova, fragments of IPVL were excised from non-GD IPVL and incubated in the *in vitro* sperm-IPVL assay (see Section 2.6.3 for method) with the same concentration of spermatozoa. A significant difference (one-way ANOVA; $P <$

0.005) was seen between the number of points of hydrolysis produced in the IPVL overlying the GD region and in non-GD IPVL in ova fertilized *in vitro*, and in isolated fragments of non-GD IPVL taken from the same ova and further incubated with spermatozoa in the *in vitro* sperm-IPVL assay (see Section 2.6.3) (Figure 22). Further statistical analysis showed a significant 7.5 fold increase (Tukey-Kramer multiple comparisons test; $P < 0.05$) in sperm hydrolytic activity towards the IPVL overlying the GD compared with areas away from this region, in ovum fertilized *in vitro*. However, when pieces of IPVL from the same fertilized ovum were removed from the non-GD region and incubated with the same concentration of spermatozoa (1.25×10^7 spermatozoa ml^{-1}) in the *in vitro* sperm-IPVL assay, the number of holes increased significantly by approximately 8 fold (Tukey-Kramer multiple comparisons test; $P < 0.01$) to numbers comparable to those at the GD region in the intact ovum. No significant difference was seen between the number of points of hydrolysis produced in the GD region after fertilization *in vitro* and the number of holes produced in non-GD regions after further incubation in the *in vitro* sperm-IPVL assay (Tukey-Kramer multiple comparisons test; $P > 0.05$).

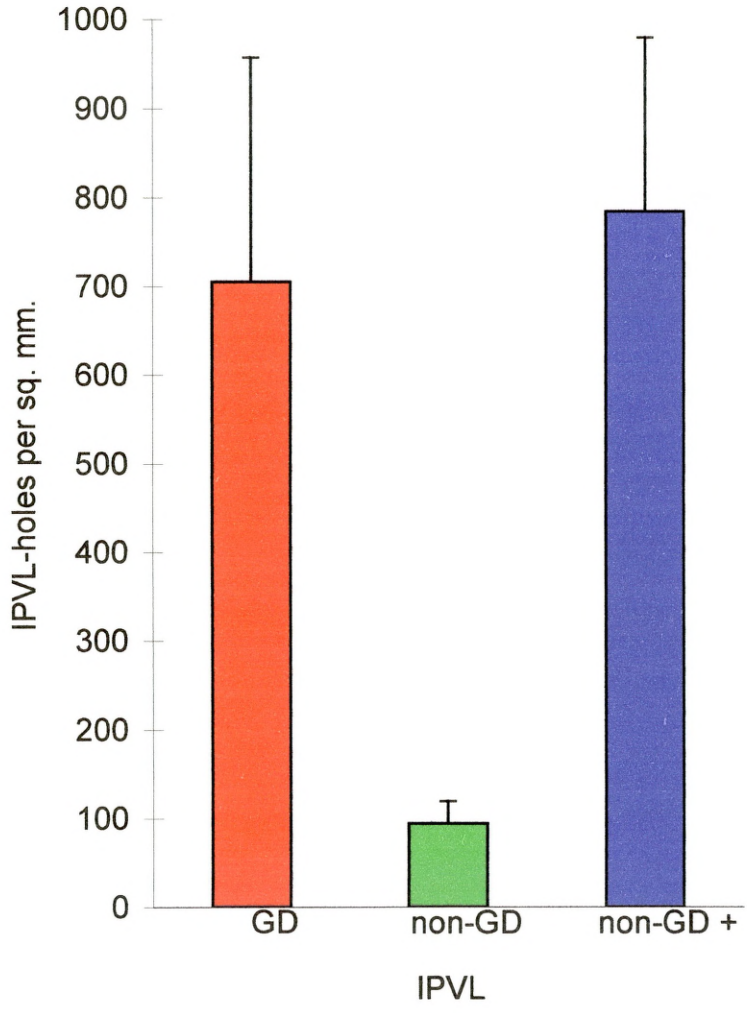


Figure 22. Points of hydrolysis produced in the IPVL overlying the GD region (GD), and at areas away from the GD (non-GD) by 1.25 x 10^7 spermatozoa ml^{-1} following *in vitro* fertilization of the intact ovum and in isolated fragments of non-GD IPVL after further incubation *in vitro* with the same concentration of spermatozoa (non-GD+). Results are means ± SD of 3 experiments.

3.6.2 Determination of the Role of Yolk in the Preferential Hydrolysis of the IPVL

A significant difference (one-way ANOVA; $P < 0.0001$) was seen in the ability of chicken spermatozoa to form points of hydrolysis in fragments of ovulated IPVL in the presence of 10% yolk material or in excised fragments of ovulated IPVL with the yolk attached, compared with control samples incubated in DMEM only (see Section 2.6.3.2 for method). However, further analysis using Dunnetts multiple comparison's test showed that following the addition of 10% egg yolk to the *in vitro* sperm-IPVL assay (see Section 2.6.3.2 for protocol) no significant difference was seen in the ability of spermatozoa to hydrolyse the IPVL compared with control samples incubated in DMEM alone (Dunnetts multiple comparison's test; $P > 0.1$) (Figure 23). Attempts to increase the concentration of yolk resulted in a decrease in sperm motility as the solution became too viscous. When pieces of IPVL were isolated with the yolk still attached and incubated with spermatozoa *in vitro*, the number of points of hydrolysis was significantly reduced compared with control samples incubated under the same conditions but washed free of yolk material (Dunnetts multiple comparison's test; $P < 0.0001$) (Figure 23).

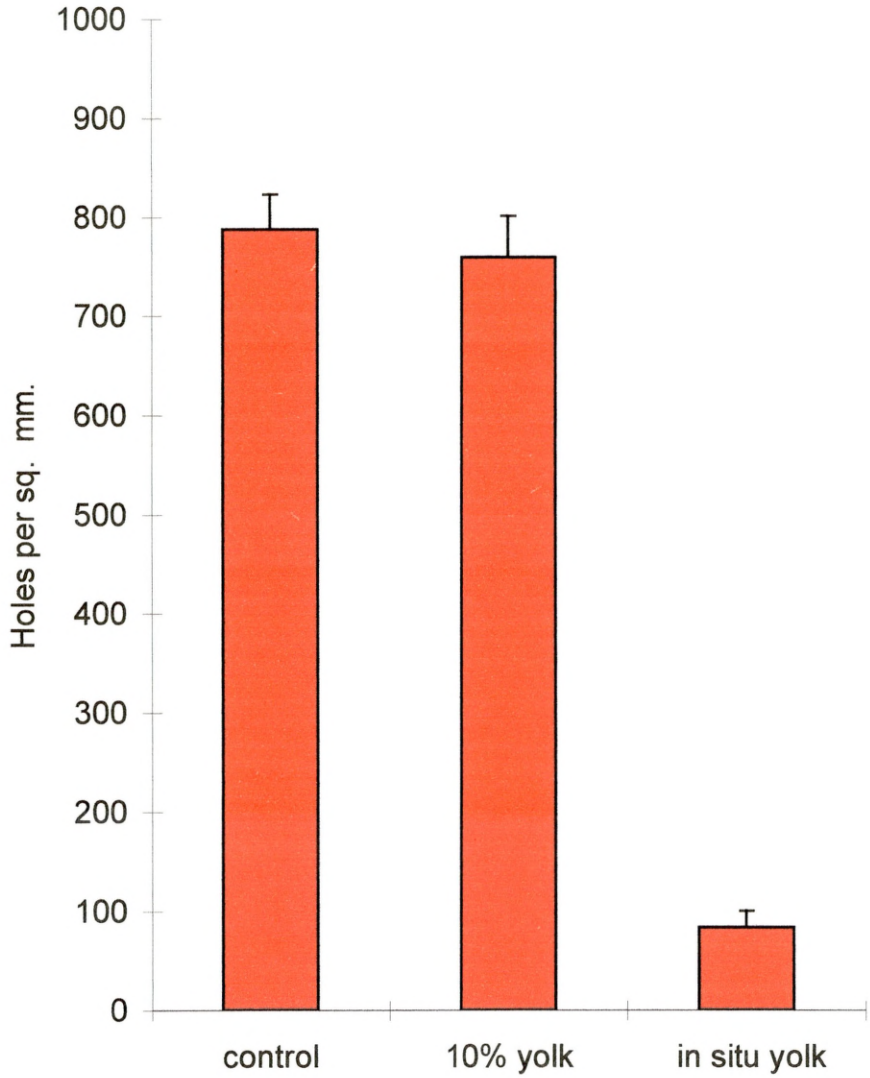
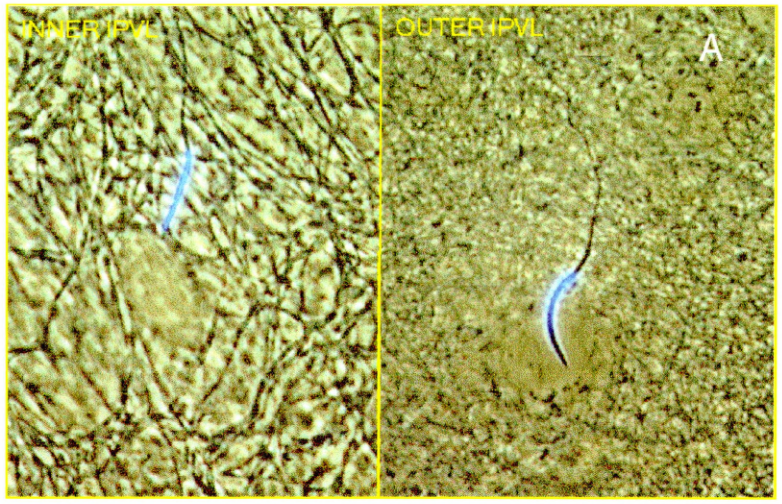


Figure 23. The effect of co-incubation of 1.25 x 10^7 spermatozoa ml^{-1} with either 10% egg yolk in the *in vitro* sperm-IPVL assay or adherent yolk material on hydrolysis the IPVL *in vitro*. Results are mean ± SD of 3 experiments.

3.6.3 Preferential Binding Of Spermatozoa to the Inner Or Outer IPVL

Microscopic analysis of the IPVL at x1000 magnification revealed that the inner and outer surfaces have a different morphology. The inner surface, which is adjacent to the yolk in the intact ovum, can be identified by its open fibrous appearance, while the outer surface, which sperm interact with *in vivo*, has a more granular appearance (Figure 24a).

Spermatozoa were found to hydrolyse both the inner and outer IPVL in the *in vitro* sperm-IPVL assay (see Section 2.6.3.1 for protocol), although the number of points of hydrolysis produced in the outer, granular surface of the IPVL was significantly greater than those produced in the inner, fibrous, surface (2 sample unpaired t-test; $P < 0.05$) (Figure 24b).



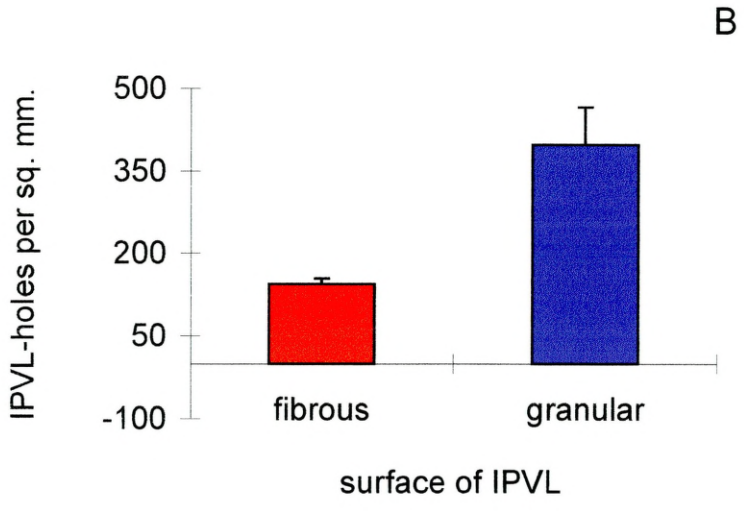


Figure 24. Preferential hydrolysis of the inner and outer surfaces of the IPVL by spermatozoa. (A) shows the Inner (fibrous) and outer (granular) surface of the IPVL with spermatozoa associated with points of hydrolysis (10 µm in diameter). (B) shows the numbers of holes produced by 1.25 x 10^7 spermatozoa ml^{-1} after exposure to either the inner surface or outer surface of the IPVL; mean ± SD of 3 experiments.

3.7 THE AVIAN ACROSOME REACTION

3.7 .1 Assessment of Methods for Detection of Acrosome Reacted Spermatozoa

Several methods used to determine the acrosomal status of spermatozoa from various animal species were investigated for their ability to detect the AR in chicken spermatozoa. Initial studies involved incubating 1.25 x 10^7 spermatozoa in 1 ml of DMEM containing homogenised IPVL for 5 min at 40°C, to induce the physiological acrosome reaction (acrosome-reacted spermatozoa). IPVL preparations were prepared by homogenising 1 IPVL from laid chicken eggs in 10 ml of DMEM. Control spermatozoa samples were incubated under the same conditions, without IPVL (acrosome-intact spermatozoa).

3.7.1.1 Simple microscopic methods

Chicken spermatozoa were incubated with homogenised preparations of IPVL (acrosome-reacted) and in DMEM alone (acrosome-intact) before examination by phase contrast and differential interference contrast (DIC) microscopy at x1000 magnification. No change was seen in the acrosomal membranes of spermatozoa from these birds. However, a small percentage (2.6 ± 2.5) of acrosome-reacted spermatozoa showed loss of the acrosome (see Figure 25) following incubation in the sperm-

IPVL assay for 5 min. (see Figure 2 a), but this was not significantly different from control samples (1.3 ± 0.8) incubated in DMEM alone (2 sample unpaired t-test; $P > 0.5$; n=6).

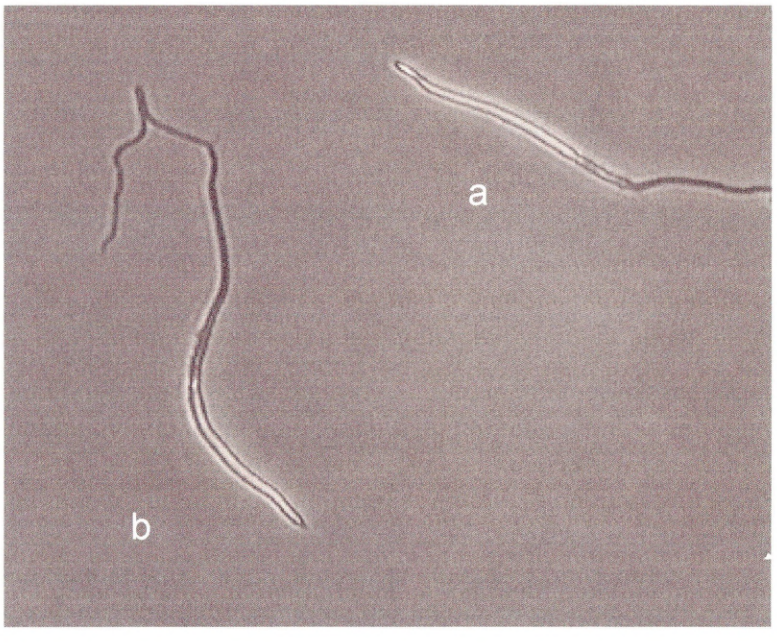


Figure 25. Phase contrast micrograph of chicken spermatozoa following incubation with IPVL at 40°C for 5 min; (a) shows spermatozoa with intact acrosome and (b) shows spermatozoa with no acrosomal tip.

3.7.1.2 Chlortetracycline (CTC) fluorescence assay

3.7.1.2.1 *Bull spermatozoa*

Cryopreserved bull spermatozoa was used to test the efficacy of chlortetracycline (CTC) as a means of detecting acrosome-reacted chicken spermatozoa (see Section 2.7.2.2). In bull spermatozoa, as in other species, three typical CTC patterns have been identified. They are: (1) 'F' pattern in which spermatozoa display an intense, uniform fluorescence over the whole head, which is characteristic of uncapacitated spermatozoa; (2) 'B' pattern in which a fluorescence-free band is seen at the postacrosomal region, which is characteristic of capacitated, acrosome-intact spermatozoa and (3) 'AR' pattern with almost no fluorescence over the acrosome except for a band at the equatorial segment, which is characteristic of acrosome-reacted sperm (Fraser *et al.*, 1995). However as bull spermatozoa were used merely to test the efficacy of the CTC assay, only the 'F' and 'AR' patterns were scored.

Uncapacitated bull spermatozoa displayed intense yellow fluorescence over the whole acrosomal region in 48.0 ± 12.8% of spermatozoa in these samples. Following capacitation (see Section 2.3.6.1 for protocol) and induction of the AR with A23187 (see Section 2.7.1.1 for protocol), the percentage of spermatozoa that displayed fluorescence over the entire acrosome was reduced to 17.7 ± 3% (2

sample unpaired t-test; $P < 0.02$; n=3). Mitochondrial staining was seen in all samples.

3.7.1.2.2 *Chicken spermatozoa*

Fresh chicken spermatozoa incubated in DMEM for 5 min showed intense staining of the mitochondria in all spermatozoa viewed and in a small region at the base of the acrosome (Figure 26) in 85 ± 9.6% of spermatozoa. After incubation of spermatozoa with homogenised preparations of IPVL to induce the AR (see Section 3.7.1), no significant change in fluorescence pattern was seen. Mitochondrial staining was again present in all spermatozoa viewed and 89 ± 6.7% (2 sample unpaired t-test; $P > 0.5$; n=4) of spermatozoa showed staining at the base of the acrosome.

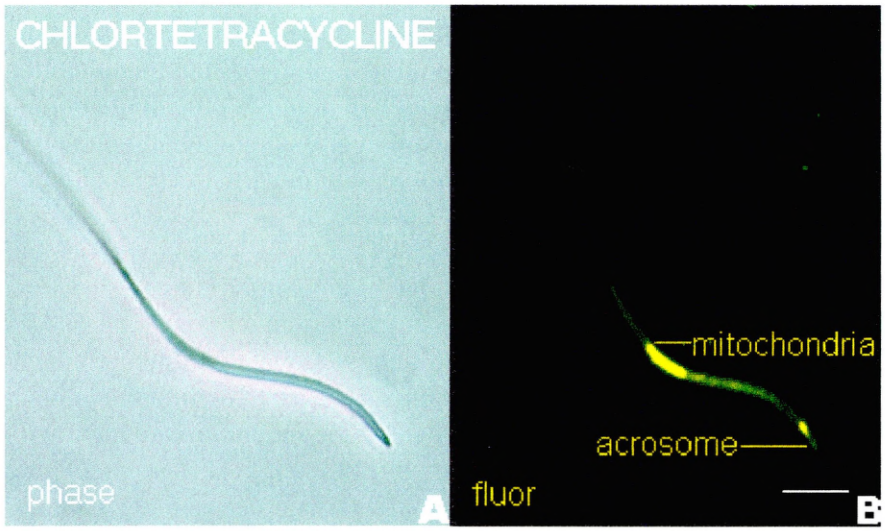


Figure 26. Normal acrosome intact chicken spermatozoa following staining with chlortetracycline (CTC). (A) shows the spermatozoa viewed using phase contrast microscopy. (B) shows the same spermatozoa viewed using epifluorescence microscopy where CTC staining can be seen at the mitochondria and at the base of the acrosome. Scale bar represents 5 µm.

3.7.1.3 Tetrazolium dye reduction assay

Following incubation of chicken spermatozoa in DMEM (acrosome-intact), or DMEM containing homogenised preparations of IPVL for 5 min at 40°C (acrosome-reacted) (see Section 3.7.1), samples were briefly microcentrifuged at 1889 g for 1 min and incubated in the tetrazolium dye reduction assay (see Section 2.4.2 for protocol) which was modified so that samples were incubated for 1 h at room temperature to increase colour intensity. Samples were briefly microcentrifuged at 1889 g and resuspended in 50 µl of NaCl-TES. A 10 µl aliquot was placed on a microscope slide, covered with a coverslip and viewed at x 1000 magnification.

In control samples, incubated in DMEM alone, dark pink spots were seen at the mitochondria and base of the acrosome in 83.8 ± 4% of spermatozoa (Figure 27). After incubation with homogenised preparations of IPVL, the number of spermatozoa displaying acrosomal staining reduced significantly to 53 ± 6% (2 sample unpaired t-test; $P < 0.005$; n=3). Mitochondrial staining was also reduced in these samples.

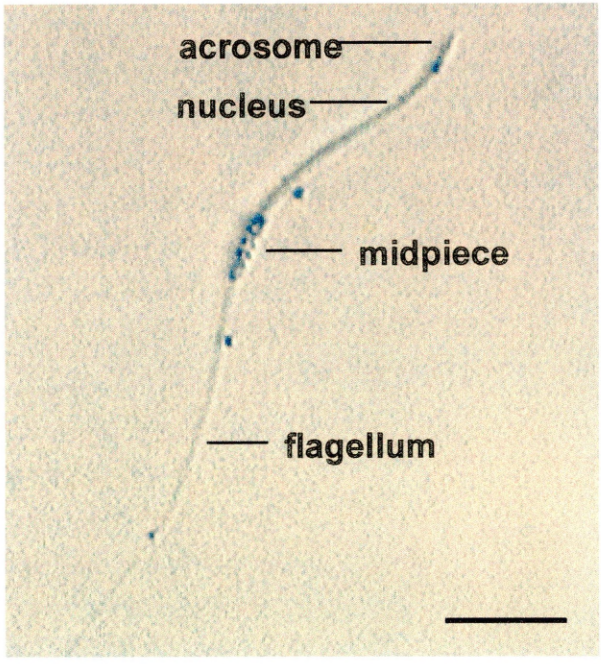


Figure 27. Normal acrosome-intact chicken spermatozoa following staining with INT-tetrazolium. The insoluble dye can be seen as dark pink spots at the mitochondria and at the base of the acrosome. Scale bar represents 5 μm.

3.7.1.4 FITC-Lectin labelling of sperm acrosomes

FITC-conjugated lectin from LTA exhibited a very weak, but fairly uniform fluorescence over the entire length of spermatozoa in samples incubated both in the presence of IPVL (acrosome-reacted) and DMEM alone (acrosome-intact) (see Section 2.7.2.3.1 for method). Similarly, a weak level of fluorescence was seen with spermatozoa incubated with FITC-Con A, but fluorescence was generally restricted to the head and midpiece regions. No change in staining pattern was detected between acrosome-intact and acrosome-reacted samples. FITC-PSA failed to label acrosome-intact spermatozoa samples. However, after incubation with homogenised preparations of IPVL, a small percentage of spermatozoa (3.7 ± 2.1%) showed a dull fluorescence over the acrosomal region, but this was not significantly different from control samples in which 1.0 ± 1% of spermatozoa were labelled with FITC-PNA (2-sample unpaired t-test; $P > 0.1$; n=3).

Intense staining of both acrosome-intact and acrosome-reacted spermatozoa was found after incubation with FITC-WGA. Staining was intense throughout the spermatozoa and extensive agglutination was seen, mainly between flagella.

Of the lectins screened, only FITC-conjugated PNA was able to reliably distinguish between acrosome-intact and acrosome-reacted chicken spermatozoa (Figure 28). Samples incubated with homogenised preparations of IPVL showed intense fluorescence over the acrosomal region in 42.9 ± 4.7 % of spermatozoa. In control samples fluorescence was largely absent, with only 4.6 ± 2.5% of acrosomes stained with FITC-PNA (2 sample unpaired t-test; $P < 0.0001$; n=6).

The majority of spermatozoa retained their acrosomes after induction of the AR with preparations of IPVL. However, a small percentage (3.6 ± 2%) of spermatozoa lost there acrosomes and the detached acrosomal tips were also found to be labelled with FITC-PNA. As these were assumed to be damaged spermatozoa, only spermatozoa with intact acrosomes were routinely scored as acrosome-reacted.

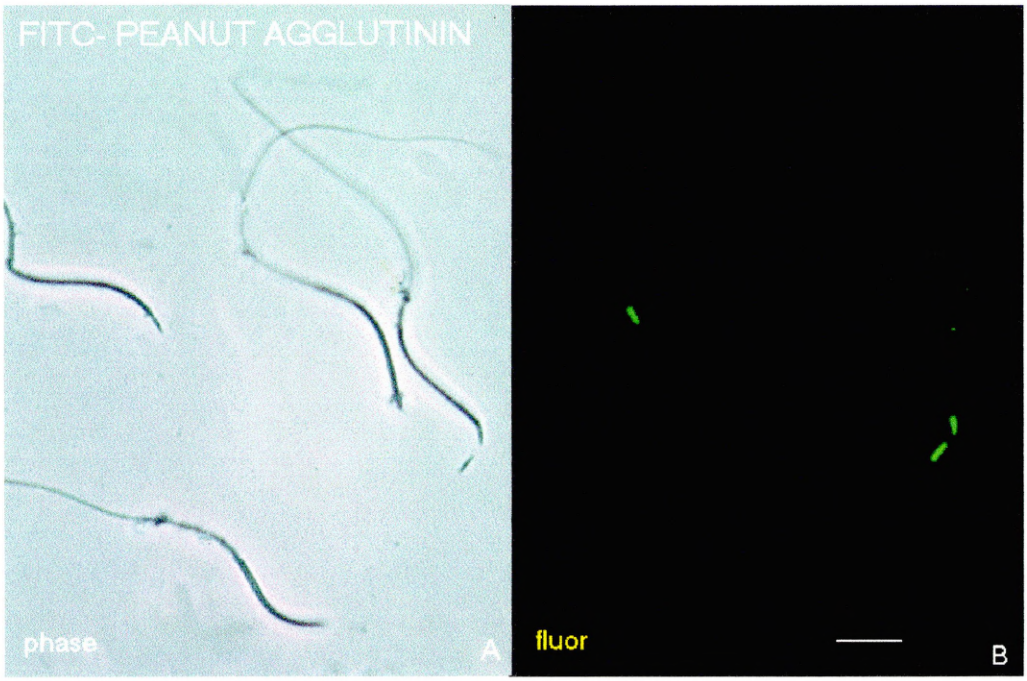


Figure 28. Chicken spermatozoa following FITC-PNA labelling of exposed acrosomes, both detached and *in situ*, following incubation with homogenised preparations of IPVL. (A) shows a population of spermatozoa using phase contrast microscopy. (B) shows the same population of spermatozoa using epilfuorescence microscopy where spermatozoa with exposed acrosomes are labelled with FITC-PNA and identified as having green acrosomal tips. Scale bar represents 5 µm.

3.7.1.4.1 *FITC-PNA labelling of chicken sperm acrosomes following incubation with solubilised IPVL*

In 3 separate experiments, IPVL was solubilised in 5 mmol l^{-1} (NaH_2PO_4) pH 2.5 for I h at 70°C, before fractionation by FPLC (see Section 2.8.1 for method). Figure 29 shows a typical protein profile obtained following FPLC. Fractions corresponding to the peaks were pooled and immediately co-incubated with 1.25 x 10^7 spermatozoa ml^{-1} for 5 min at 30°C (see Section 2.7.3 for method) and assessed for AR inducing activity using FITC-PNA labelling of exposed acrosomes (see Section 2.7.2.3.1 for method). For each experiment 3 separate FPLC runs were carried out.

A fraction corresponding to peak 1, of apparent molecular weight 42 kDa, induced the AR in chicken spermatozoa, resulting in a significant increase in FITC-PNA labelling (see Section 2.7.2.3.1 for protocol), of exposed acrosomes to 50.1 ± 3.5 %, compared with 1.8 ± 1.6 % in control samples (2 sample un-paired T-test; $P < 0.01$; n=3). No significant increase in FITC-PNA labelling of sperm acrosomes was seen in the fractions corresponding to peaks 2 to 6, compared with spermatozoa incubated in NaCl-TES alone (each 2 sample un-paired T-test; $P > 0.05$; n=3). Solubilised IPVL samples had to be immediately assessed for AR inducing activity following fractionation by FPLC as the IPVL proteins

quickly re-aggregated. Protein concentrations were below the level of detection by the BCA assay.

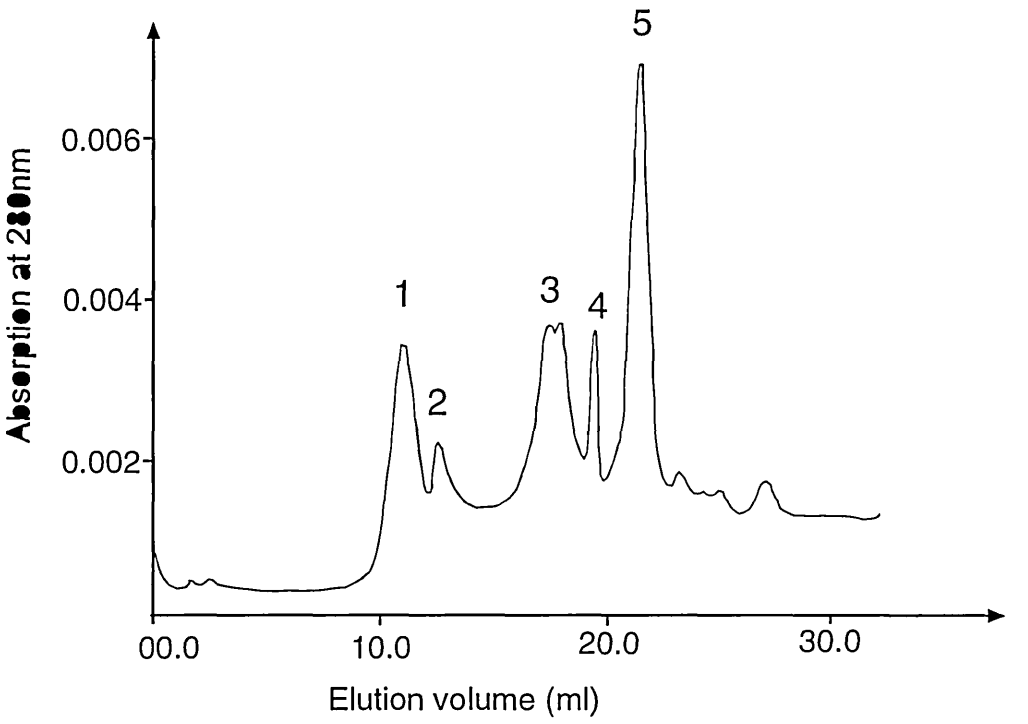


Figure 29. Typical protein profile obtained following fractionation of solubilised IPVL. Six distinct peaks of absorbance at 280 nm were detected.

3.7.2 Non Physiological Methods of Exposing Chicken Sperm Acrosomal Membranes

3.7.2.1 A23187

Incubation of chicken spermatozoa in the presence of the Ca^{2+} ionophore A23187, failed to induce the AR in chicken spermatozoa (see Section 2.7.1.1 for method). After as little as 5 min in these conditions, sperm viability was severely affected, as assessed by the tetrazolium dye reduction assay (see Section 2.4.2 for method). A23187 treated spermatozoa produced very little colour change in the medium and when viewed under the microscope, 31.3 ± 11.4% of spermatozoa had no spots of metabolic activity at either the acrosome or mitochondria, compared with control samples at 3.0 ± 1.9 (2 sample unpaired T-test; $P < 0.05$). Those that remained in the treated samples showed a decrease in the depth of colour intensity in all spots present. Assessment of acrosomal status using FITC-PNA showed no significant difference in the percentage of labelled acrosomes between control samples at 3.5 ± 0.6% and A23187 treated sperm samples at 3.7 ± 1.1% (2 sample unpaired t-test; $P > 0.5$; n=3).

3.7.2.2 Sonication and detergent treatment

Spermatozoa were disrupted by mild sonication and detergent treatment in order to expose FITC-PNA binding sites on sperm acrosomes. Results were compared with spermatozoa incubated with homogenised preparations of IPVL. Following these treatments, the general integrity of the sperm plasma membrane was assessed using aniline-eosin smears (see Section 2.4.1 for method).

3.7.2.2.1 *Aniline-eosin assessment of spermatozoa membranes*

A one-way ANOVA showed a significant difference ($P < 0.0001$) in the ability of different concentrations of digitonin to permeabilise the plasma membrane of chicken spermatozoa as assessed by the aniline-eosin dye exclusion assay (see Section 2.4.1 for method). At concentrations of less than 25 µg ml^{-1} digitonin, permeabilisation of plasma membranes was not significantly increased compared with control samples (Dunnett's multiple comparison's test; $P > 0.05$). At 25 µg ml^{-1} digitonin, a significant decrease in spermatozoa showing intact plasma membranes was seen, although the general integrity of the sperm was not compromised (Dunnett's multiple comparison's test; $P < 0.01$). A further increase in detergent concentration to 50 µg ml^{-1} digitonin resulted in permeabilisation of more than 80% of spermatozoa plasma membranes

(Dunnett's multiple comparison's test; $P < 0.01$) but several spermatozoa had lost their flagella. At concentrations above 50 μg ml^{-1} digitonin, less than 5% of spermatozoa showed intact plasma membranes (Dunnett's multiple comparison's test; $P < 0.01$) and the spermatozoa were largely fragmented (see Figure 30).

Following sonication of spermatozoa for 1, 2 or 3 s pulses, aniline-eosin smears showed no morphologically normal spermatozoa. At sonication times greater than 1s spermatozoa were very broken up, many with detached heads and tails, therefore were not used for further studies.

Incubation of spermatozoa with homogenised preparations of IPVL (see Section 3.7) resulted in a significant increase in the permeability of chicken spermatozoa to eosin dye (2 sample unpaired t-test; $P < 0.0001$). Figure 30 shows a histogram representing the percentage of morphologically normal spermatozoa following sonication for 1 s and incubation with preparations of IPVL.

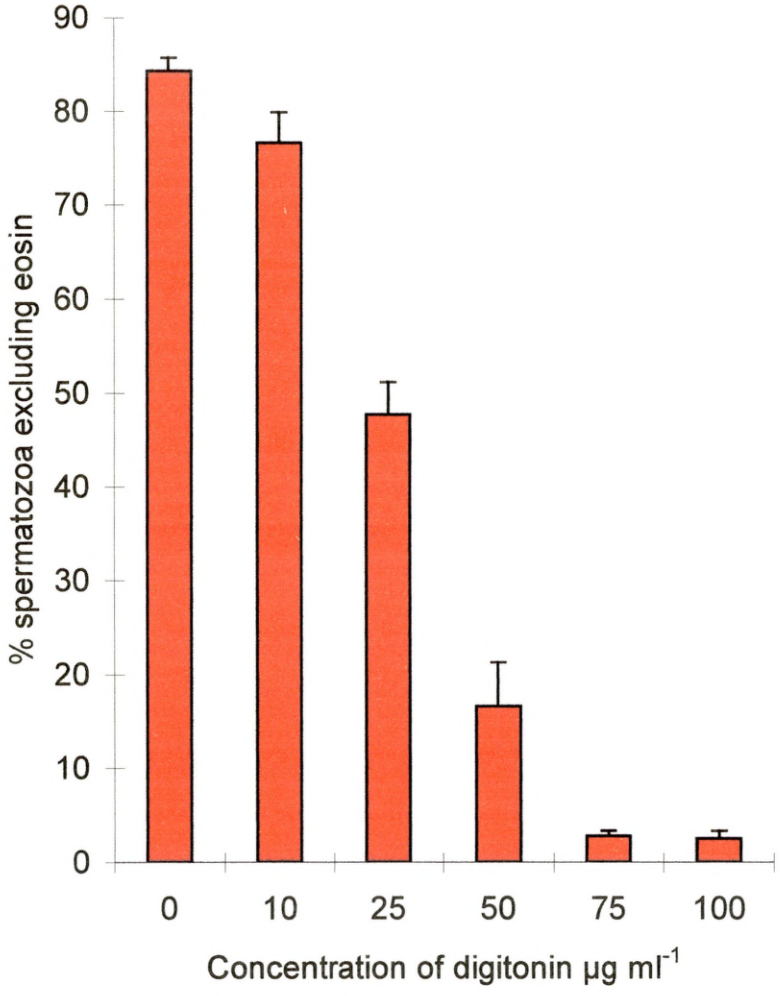


Figure 30. Percentage of chicken spermatozoa showing intact plasma membranes following incubation with various concentrations of digitonin, as determined by aniline-eosin smears, Results are mean ± SD of 3 experiments.

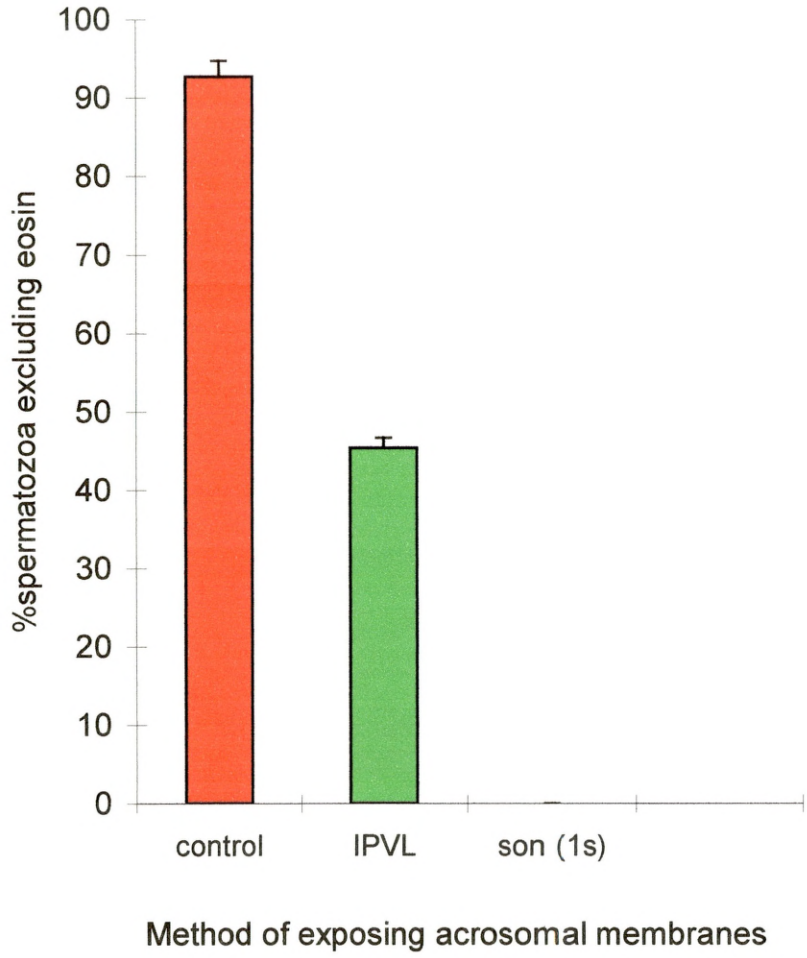


Figure 31. Percentage of chicken spermatozoa excluding eosin dye following incubation with preparations of IPVL and sonication (son) for 1s. Results are mean ± SD of 3 experiments.

3.7.2.2.2 *Assessment of exposed acrosomes by FITC-PNA labelling*

As spermatozoa Incubated with 25 and 50 µg ml^{-1} digitonin showed disruption of the plasma membrane but still retained some structural integrity, spermatozoa were incubated in these concentrations of detergent before assessment of acrosomal status using FITC-PNA. A one-way ANOVA showed significant differences in the number of FITC-PNA labelled acrosomes between 0, 25 and 50 µg ml^{-1} digitonin treated samples ($P < 0.001$; n = 3). However, further analysis; showed no significant differences between 0 and 25 µg ml^{-1} digitonin samples (Dunnett's multiple comparison's test; $P > 0.05$; n = 3), but a significant difference between control and 50 µg ml^{-1} digitonin samples (Dunnett's multiple comparison's test; $P < 0.01$; n = 3)

Exposure of acrosomal membranes by mild sonication for 1s resulted in a significant increase in FITC-PNA labelled acrosomes compared with control samples (2 sample unpaired T-test; $P < 0.04$; n=3).

Incubation of spermatozoa with homogenised preparations of IPVL resulted in a significant increase in FITC-PNA labelled acrosomes compared with control samples incubated in DMEM only (2 sample unpaired T-test; $P < 0.02$; n=3). Figure 32 shows the percentage of chicken spermatozoa with FITC-PNA labelled acrosomes following the various disruption methods.

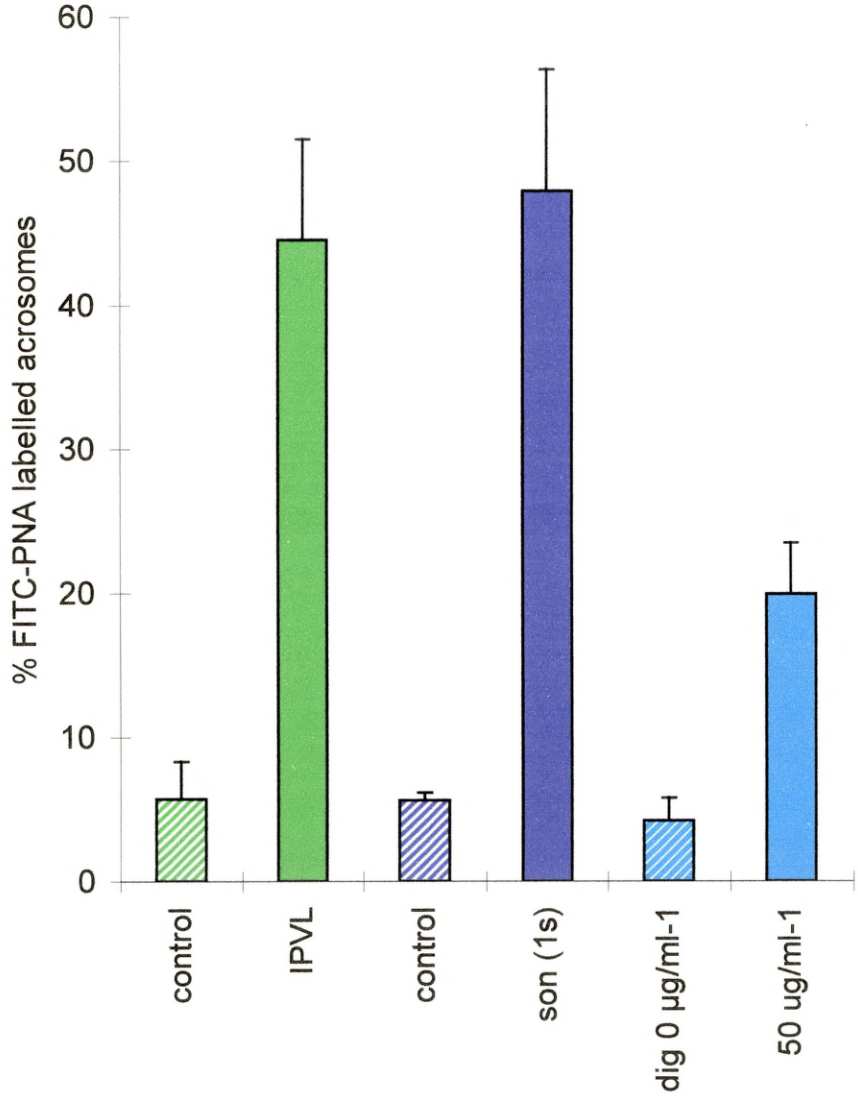


Figure 32. Percentage of chicken spermatozoa acrosomes labelled with FITC-PNA following various disruption methods. Results are mean ± SD of 3 experiments.

3.7.3 Examination of Colloidal Gold-Conjugated-PNA Labelling of Chicken Sperm Acrosomes by Transmission Electron Microscopy

To determine the site of PNA binding to the acrosomes of chicken spermatozoa, samples were incubated both in the presence and absence of IPVL before being labelled with colloidal gold-PNA for examination by transmission electron microscopy (TEM) (see Section 2.7.2.3.2 for method). Figure 33 shows (a) spermatozoa incubated in DMEM in the absence of IPVL (acrosome-intact) and (b) spermatozoa incubated in DMEM in the presence of IPVL (acrosome-reacted). Interpretation of the electron micrographs indicates that in chickens, the acrosome is bounded by the outer acrosomal and plasma membranes. The IAM appears to be continuous with the OAM and is located at the surface opposed to the perforatorium of acrosome-intact spermatozoa. Acrosome-intact spermatozoa (Figure 33a) were not labelled with colloidal gold-PNA. Acrosome reacted spermatozoa (i.e. those that had been incubated with the IPVL), appeared to have no outer acrosomal or plasma membranes and the exposed acrosomes were labelled with colloidal gold-PNA.

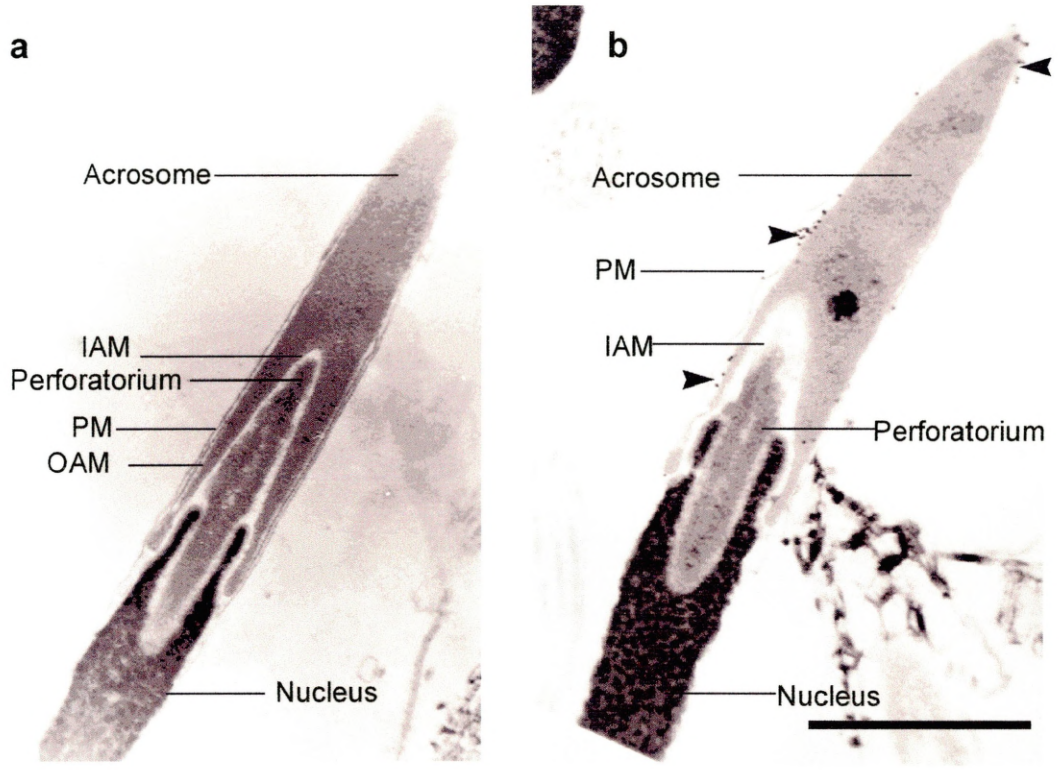


Figure 33. Transmission electron micrographs of an intact acrosome (a) and exposed acrosome (b) from chicken spermatozoa showing the acrosome, plasma membrane (PM), outer acrosomal membrane (OAM), inner acrosomal membrane (IAM), perforatorium and nucleus. Colloidal gold-PNA particles (depicted as arrow-heads) can be seen bound only to the exposed acrosomes of spermatozoa (b) and not to acrosome-intact spermatozoa (a). Scale bar represents 0.5 µm.

3.7.4 The Effect of Calcium and Temperature on Chicken Spermatozoa Acrosome Reaction Detected by FITC-PNA Labelling

A significant difference was seen in the ability of chicken spermatozoa to undergo the AR in the presence of extracellular Ca^{2+} at 30°C and 40°C (one-way ANOVA; $P < 0.0001$). At 30°C chicken spermatozoa required only the presence of the IPVL to undergo the AR. No significant difference was found in the number of FITC-PNA labelled acrosomes in samples incubated in NaCl-TES with the addition of 5 mmol l^{-1} Ca^{2+} and those incubated in NaCl-TES with 1 mmol l^{-1} EGTA, to remove extracellular calcium (Tukey-Kramer multiple comparisons test; $P > 0.05$) (Figure 34). However, at 40°C extracellular calcium was required for spermatozoa to undergo the AR. Removal of Ca^{2+} from the medium by the addition of 1 mmol l^{-1} EGTA resulted in a significant decrease in FITC-PNA labelled acrosomes (Tukey-Kramer multiple comparisons test; $P < 0.01$). No significant difference was seen in the ability of chicken spermatozoa to under go the AR between samples incubated at 40°C with extracellular Ca^{2+} and samples incubated at 30°C either with or without extracellular Ca^{2+} (Tukey-Kramer multiple comparisons test; both $P > 0.05$).

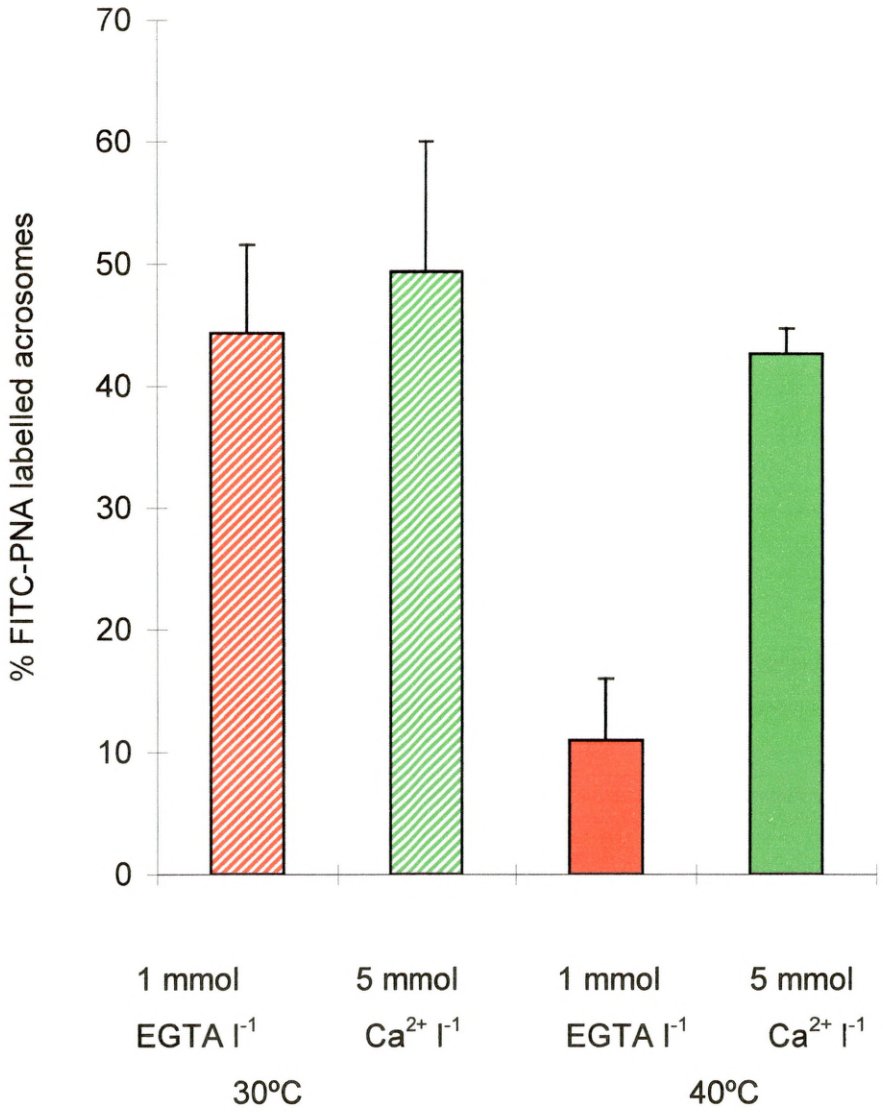


Figure 34. The effect of temperature and calcium on the ability of chicken spermatozoa to undergo the acrosome reaction. 1.25 x 10^7 spermatozoa ml^{-1} were incubated in the presence of 1 mmol EGTA l^{-1} ■ or 5 mmol Ca^{2+} l ■. Results are mean ± SD of 3 experiments.

3.7.5 Assessment of FITC-PNA Labelling as a Means of Detecting Acrosome Reacted Turkey and Quail Spermatozoa

Spermatozoa from chicken, turkey and quail showed intense FITC-PNA staining of acrosomes in samples incubated for 5 min at 40°C with homologous preparations of IPVL in DMEM (Figure 35). In all cases there was a significant increase in the number of FITC-PNA labelled acrosomes in samples incubated with IPVL compared with control samples incubated in DMEM alone (one-way ANOVA; $P < 0.001$). Acrosome-reacted spermatozoa from chicken, turkey and quail showed a similar affinity for FITC-PNA and no significant difference was seen between the three species, in the percentage of fluorescent acrosomes visualised (Tukey-Kramer multiple comparisons test; all $P >. 0.05$) (Figure 36).

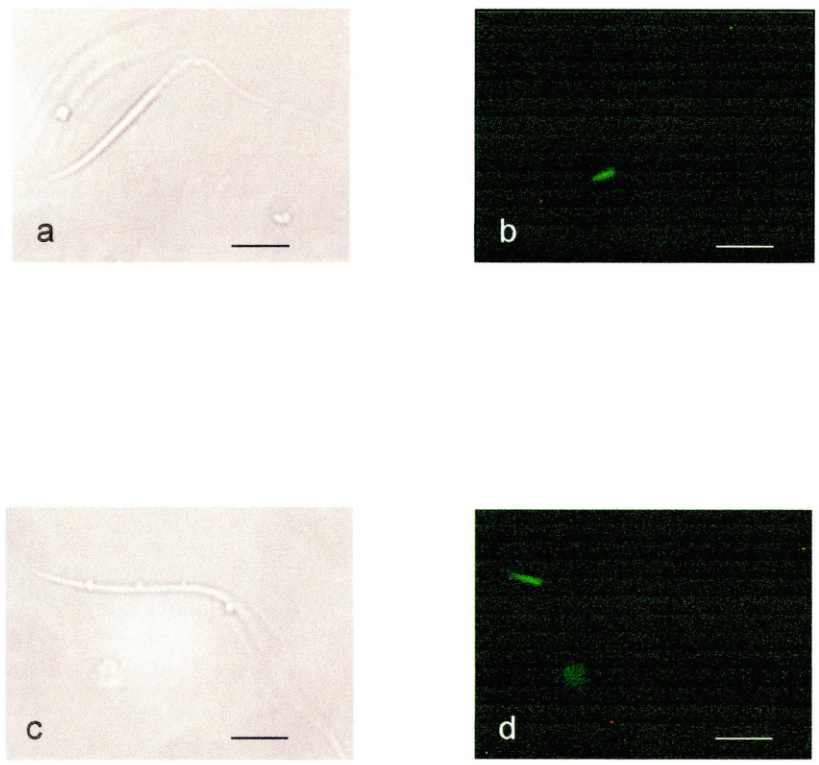


Figure 35. FITC-PNA staining of exposed acrosomes in turkey and quail spermatozoa following incubation with homologous IPVL, using phase contrast (a; turkey), (c; quail) and epifluorescence (b, turkey), (d, quail) scale bar represents 5 µm.

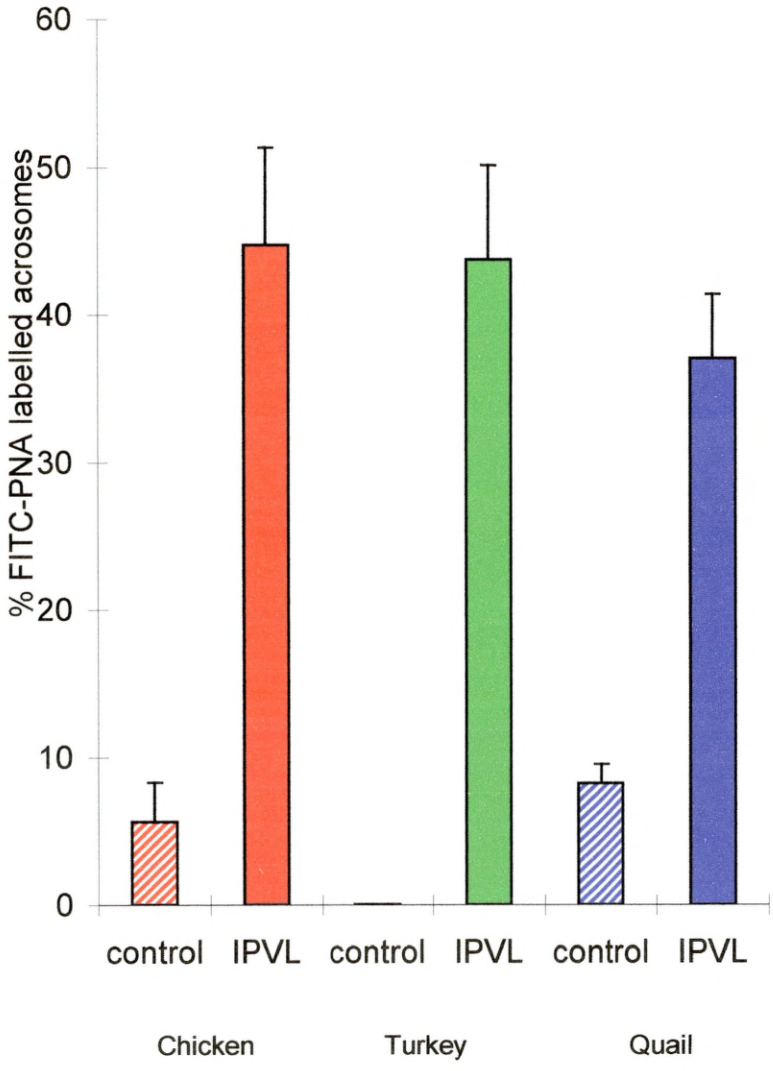


Figure 36. Percentage of FITC-PNA labelled acrosomes in spermatozoa samples incubated with homologous IPVL (acrosome-reacted) compared with control samples (acrosome-intact) in chicken, turkey and quail. Results are mean ± SD of 3 experiments.

3.8 DETERMINATION OF THE ROLE OF CARBOHYDRATES IN SPERM-IPVL INTERACTION IN BIRDS

Assessment of the role of carbohydrates in sperm-IPVL interaction in chickens was evaluated using lectins as sugar-specific markers to determine the availability of sugars on intact sheets of IPVL from laid eggs. The *in vitro* sperm-IPVL assay was used to identify specific saccharides involved in the interaction of spermatozoa with the ovum using lectins and saccharides that have been implicated in sperm-zona binding in several mammalian species. The ability of O- and N- linked glycans to inhibit sperm hydrolysis of the IPVL was assessed and preliminary experiments were carried out into their role in induction of the AR.

3.8.1 Lectin Binding to IPVL from Laid Chicken Eggs

3.8.1.1 Fluorescent lectin labelling of IPVL

Several fluorescent-labelled lectins were screened for their ability to bind to the IPVL from laid chicken eggs (see Section 2.5.5 for method). In each experiment lectin binding was assessed using IPVL from the same laid egg. The efficiency of lectin binding to the layer was determined by the intensity of the fluorescent staining on a subjective scale from 0 to XXXXX (see Table 2).

Lectin	Fluorescent lectin (10 µg ml^{-1}) labelling of IPVL (n =3)	IPVL holes (as a percentage of control) (* = 100 µg ml^{-1} † = 10 µg ml^{-1})	No. of IPVLs examined in sperm-IPVL assay
WGA	XXXXX	* 0.00 ± 0 † 0.002± 0.005	8 6
S-WGA	XXXXX	* 0.00 ± 0 † 0.001± 0.002	8 6
Con A	XXX	* 73.19 ± 6.8 † 88.96 ± 10.0	8 6
PNA	XXX	* 70.1 ± 6.37 † 82.79 ± 6.72	8 6
PSA	XXX	* 76.7 ± 7.57 † 80.66 ± 8.89	8 6
UEA II	XX	* 52.75 ± 7.05	8
STA	X	* 61.94 ± 17.71 † 59.07 ± 16.0	8 6
LTA	X	* 66.53 ± 11.01	8 6

Table 2. FITC-lectin labelling of IPVL separated from laid chicken eggs and the effect of pre-incubation of the IPVL with unlabelled lectin on the ability of spermatozoa to hydrolyse the layer. The intensity of fluorescent staining was determined visually and graded on a 0 - XXXXX scale, where 0 represents no fluorescence and XXXXX represents intense fluorescence.

WGA (Figure 37) and Succinyl-WGA (S-WGA) both labelled the chicken IPVL intensely and no difference was seen between the 2 forms of the lectin in spite of slight differences in binding specificities (see Table 2 for fluorescence intensity; Appendix D for carbohydrate binding specificities). A moderate fluorescence was observed with Con A, PNA and PSA and to a slightly lesser extent, UEA II. Very little fluorescence was seen following incubation of the IPVL with STA and LTA. All lectins appeared to stain the IPVL uniformly and no difference was seen in the ability of the lectins to bind to the granular (outer) or fibrous (inner) surface of the layer.

3.8.1.2 Assessment of the ability of spermatozoa to hydrolyse the IPVL following pre-incubation with lectins

The effect of lectin binding to the IPVL on sperm-IPVL interaction was assessed using the *in vitro* sperm-IPVL assay (see Section 2.6.3.4 for method). Following pre-incubation of pieces of IPVL from the same laid egg with various lectins at 100 µg ml^{-1}, a one-way ANOVA showed a significant difference ($P < 0.0001$) in the ability of spermatozoa to hydrolyse the layer. Further analysis using the Dunnett multiple comparisons test showed there was a significant decrease in the ability of spermatozoa to hydrolyse the IPVL following pre-incubation of the layer

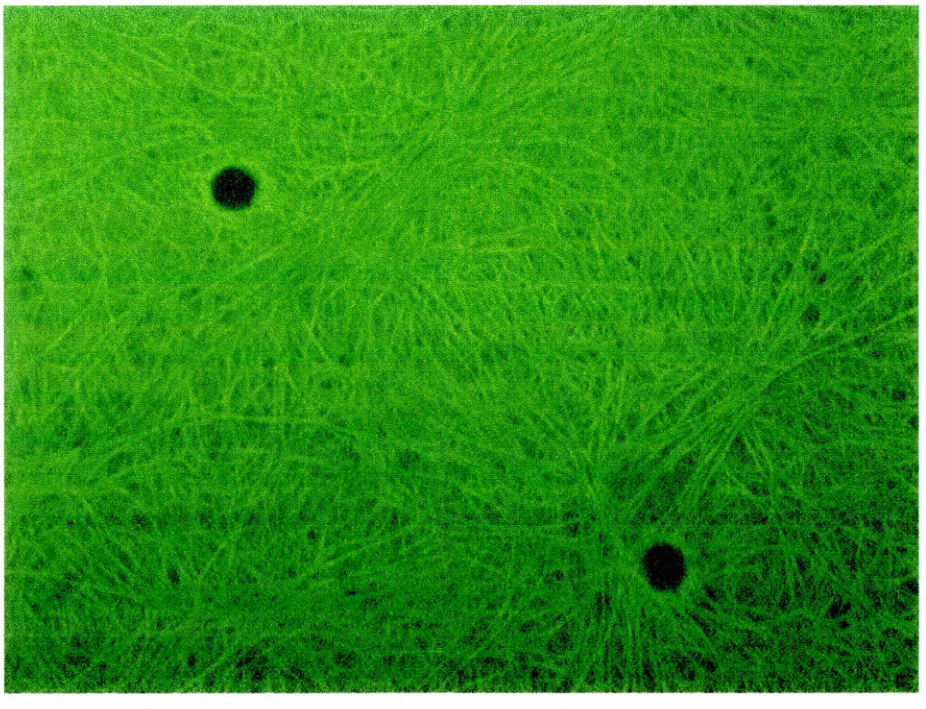


Figure 37. FITC-WGA labelling of chicken IPVL showing holes produced by chicken spermatozoa *in vitro*. IPVL-holes are 10 µm in diameter.

of the IPVL by spermatozoa ($P < 0.01$) (see Table 2). STA and LTA reduced hydrolysis of the IPVL by approximately 40% compared with control IPVL samples and Con A, and PNA, by approximately 30% (all $P < 0.01$). PSA showed the least ability to inhibit hydrolysis of the IPVL with the percentage of holes being reduced by approximately 25% of control IPVL ($P < 0.05$).

A reduction in lectin concentration to 10 μg ml^{-1} also showed a significant difference in the ability of WGA, Con A, PSA, PNA and STA to inhibit hydrolysis of the IPVL by spermatozoa (one-way ANOVA; $P < 0.0001$) although, no significant difference was seen in the number of IPVL-holes following pre-incubation with Con A, PSA, PNA or STA (Dunnett multiple comparisons test; $P > 0.05$). However, a significant decrease in hydrolysis of the IPVL by spermatozoa was observed following pre-incubation with WGA and S-WGA ($P < 0.01$). At this concentration a small number of holes were identified, but this represented only 0.002% of control samples.

3.8.2 Assessment of the Ability of WGA to Inhibit Hydrolysis of Chicken IPVL Following Pre-incubation of WGA with *N,N'*-Diacetylchitobiose

The inhibitory effect of WGA on hydrolysis of the IPVL by spermatozoa was reversed by pre-incubation of the lectin with *N,N'*-Diacetylchitobiose (see Section 2.6.3.5 for method). A one-way ANOVA showed a significant difference in the ability of spermatozoa to hydrolyse the layer following exposure of the IPVL to WGA, or WGA pre-incubated with *N,N'*-Diacetylchitobiose at 0.2 mmol l^{-1} and 0.04 mmol l^{-1}, compared with control, untreated IPVL (Table 2) ($P < 0.001$). Hydrolysis of the IPVL by spermatozoa was completely inhibited following pre-incubation of the layer with 100 µg ml^{-1} WGA, compared with control IPVL, which was not exposed to the lectin (Dunnett multiple comparisons test; $P < 0.01$). However, following pre-incubation of WGA with 0.2 mmol l^{-1} *N,N'*-Diacetylchitobiose the inhibitory effect of the lectin was reversed and the percentage of IPVL holes increased to approximately 90% of control samples (Dunnett multiple comparisons test; $P < 0.5$). When the concentration of *N,N'*-Diacetylchitobiose was reduced to 0.04 mmol l^{-1} the pre-treated WGA reduced hydrolysis of the IPVL by approximately 40% of the untreated control IPVL samples (Dunnett multiple comparisons test; $P < 0.01$).

Concentration of WGA ($\mu g\ ml^{-1}$)	Concentration of *N,N*-Diacetylchitobiose ($mmol\ l^{-1}$)	IPVL-holes as a percentage of control (%)
0	0.0	100
100	0.0	0
100	0.04	40.10 ± 5.16
100	0.2	89.87 ± 8.72

Table 3. The effect of pre-incubation of WGA with *N,N'*-Diacetylchitobiose, prior to the pre-treatment of IPVL with WGA on the ability of spermatozoa to hydrolyse the IPVL. Results are mean ± SD of 3 experiments.

3.8.3 Effect of Saccharides on Sperm-IPVL Hydrolysis *In Vitro*

The polysaccharide fucoidin and several monosaccharides were added to the *in vitro* sperm-IPVL assay to determine their effects on sperm-IPVL interaction in chickens. Fucoidin was added to a final concentration of 1 mg ml^{-1} and the monosaccharides to a final concentration of 0.1 mol l^{-1} or 0.075 mol l^{-1} for D-GlcNAc (see Section 2.6.3.3 for method). Sperm motility was not noticeably impaired at the concentrations used.

In each experiment IPVL from the same laid egg was used. A one-way ANOVA showed a significant difference in the ability of spermatozoa to form points of hydrolysis following the addition of these sugars. Further analysis using Dunnetts multiple comparisons test, showed no significant difference ($P > 0.05$) in the ability of D-glu, D- gal, D-fuc or L-fuc (all at 0.1 mol l^{-1}) to inhibit hydrolysis of the IPVL by spermatozoa. A small, but significant increase was seen in the number of IPVL-holes after the addition of D-man to the sperm-IPVL assay ($P < 0.01$). Fucoidin at 1 mg ml^{-1} and 0.075 mol l^{-1} D-GlcNAc both inhibited sperm hydrolysis of the IPVL (each $P < 0.01$). However, fucoidin reduced sperm hydrolysis of the IPVL by less than 25%, whereas D-GlcNAc dramatically inhibited the formation of points of hydrolysis by more than 70% (see Table 4).

3.8.4 Effect of D-GlcNAc on Induction of the Acrosome Reaction

Co-incubation of spermatozoa with 0.075 mol D-GlcNAc l^{-1} did not induce the AR in chicken spermatozoa and no significant difference was seen in the percentage of FITC-PNA labelled acrosomes between control samples, incubated in DMEM, at 1.6% and samples incubated with the sugar at 2% (2 sample unpaired t-test; $P > 0.5$; n=3).

Saccharide	Concentration	IPVL holes (as a percentage of control)
D-glucose (D-glc)	0.1 M	91.1 ± 7.85
D-galactose (D-gal)	0.1 M	95.33 ± 10.33
D-fucose (D-fuc)	0.1 M	93.79 ± 5.65
L-fucose (L-fuc)	0.1 M	86.35 ± 16.41
D-mannose (D-man)	0.1 M	113.78 ± 12.46
D-N-acetyl-glucosamine (D-GlcNAc)	0.075 M	26.63 ± 5.52
Fucoidin	0.1 mg ml^{-1}	76.93 ± 6.11

Table 4. The effect of saccharides on the ability of spermatozoa to hydrolyse the IPVL from laid chicken eggs in the *in vitro* sperm-IPVL assay. Results are mean ± SD from 6 experiments.

3.8.5 Effect of Endo-Glycosidase Treatment on Sperm:IPVL Interaction *In Vitro*

Pieces of follicular IPVL were incubated with equivalent activities of O- and N- glycosidases (see Section 2.8.3 for method). Following glycosidase treatment the IPVL was cut into 3 before co-incubating 2 pieces with spermatozoa in the sperm-IPVL assay (see Section 2.6.3 for method), the third piece was subjected to SDS-PAGE (see Section 3.8.5.1). No significant difference was seen in the ability of spermatozoa to hydrolyse the IPVL following O-glycosidase treatment (2 sample unpaired T-test; $P > 0.5$) compared with IPVL incubated in buffer only (Figure 38). However, the ability of spermatozoa to form points of hydrolysis in the IPVL following treatment with PNGase F to remove N-linked glycans was drastically reduced compare with control samples (2 sample unpaired T-test; $P < 0.0001$).

The orcinol sugar assay was used to ensure that sugars were being removed from the IPVL (see 2.10 for method). Following incubation of the medium removed from sheets of IPVL treated with O-glycosidases, an orange coloured medium was produced in which the total sugar content was found to be 25.53 ± 8.1 µg per 1.5 cm x 1.0 cm sheet of IPVL. Removal of N-linked glycans resulted in a yellow coloured medium following the orcinol sugar assay and the sugar content was found to be 34.99 ± 8.1 µg per sheet of IPVL.

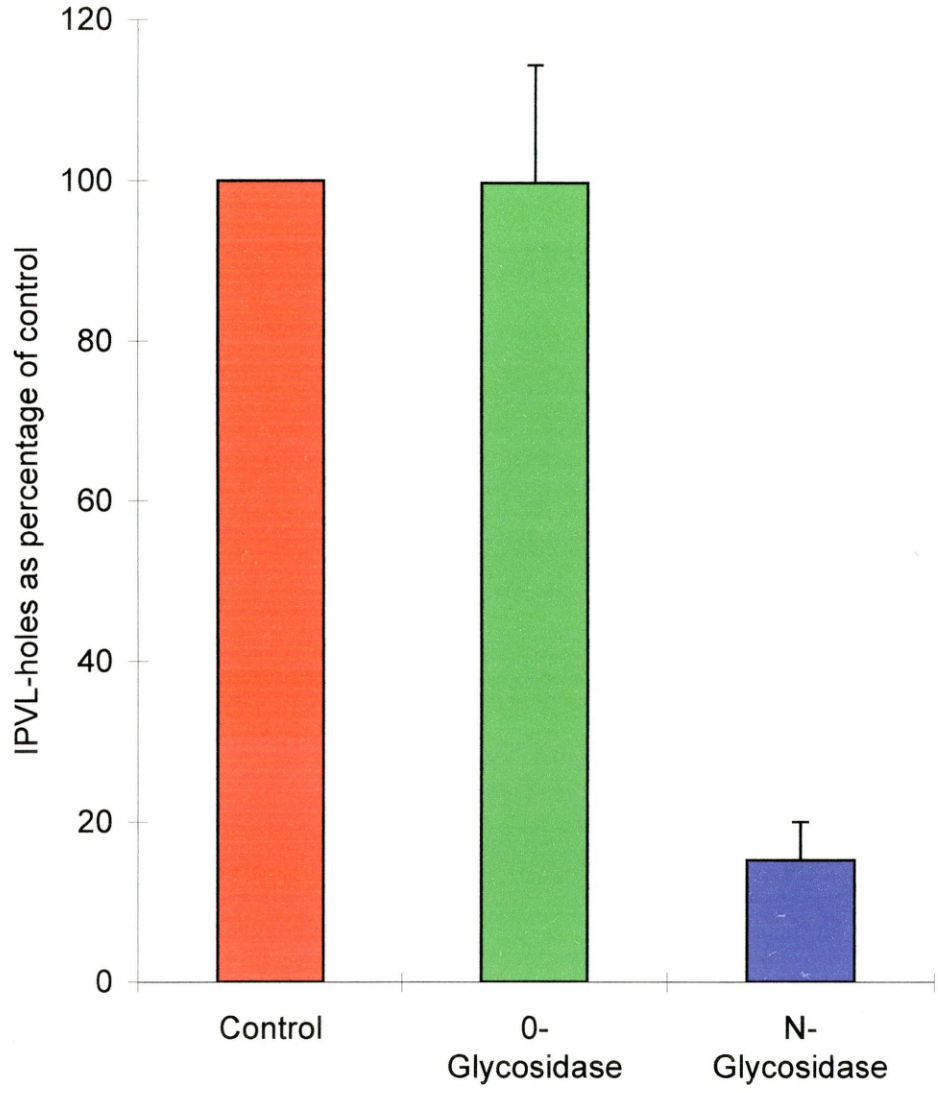


Figure 38. Effect of the selective removal of O-glycans and N-glycans from the IPVL of follicular ova on the ability of spermatozoa to form points of hydrolysis in the IPVL *in vitro*. Results are mean ± SD of 4 experiments.

3.8.5.1 Electrophoretic profiles of chicken IPVL following glycosidase treatment

Protein profiles obtained from follicular IPVL incubated with equal activities of O- and N- glycosidases (see Section 3.8.5) following SDS-PAGE are shown in Figure 39. Control samples incubated under the same conditions as glycosidase treated IPVL (O- and N-), revealed the presence of both 78 kDa and 39 kDa bands as found before for follicular IPVL (see Section 3.2). No change in mobility was seen in either of the protein bands following incubation in the presence of PNGase F (N+). However, fragments of IPVL incubated with Endo-∝-N-acetylgalactosaminidase, to remove O- linked glycans resulted in a small decrease in size in the 39 kDa band by approximately 0.5 kDa (O+). No change was seen in the 78 kDa band in samples treated with endo-∝-N-acetylgalactosaminidase.

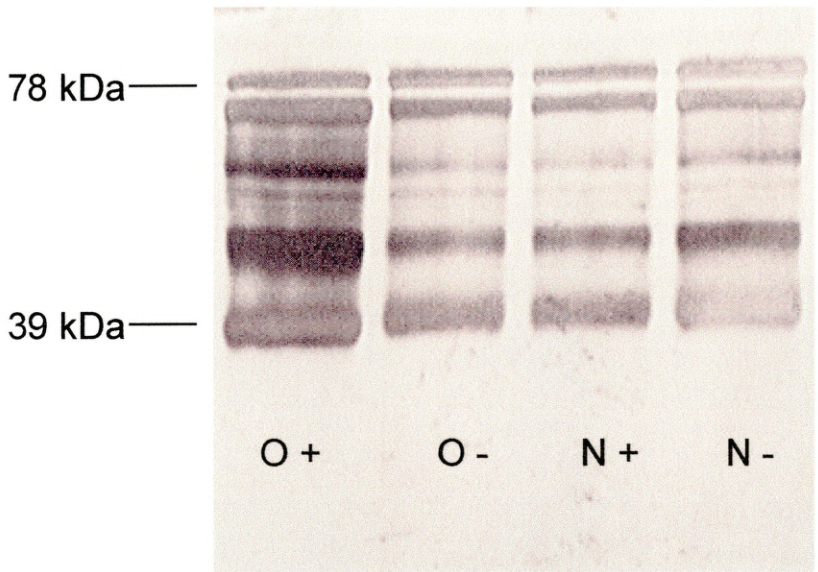


Figure 39. SDS-PAGE profiles of glycosidase treated IPVL. Follicular IPVL was treated with (O+) or without (O-) ∝-N-acetylgalactosaminidase in the presence of sialidase, fucosidase and galactonolactone to remove O-linked glycans and with (N+) or without (N-) peptide-N-glycosidase F to remove N-linked glycans. In each case 3.5 µl of protein, taken from 0.5 cm x 0.5 cm squares of IPVL, was loaded to each gel. Protein bands were visualised by silver staining after the method of Blum *et al.*, 1987.

3.8.6 The Effect of O- and N-linked Glycans Removed from the IPVL on Induction of the Acrosome Reaction in Chicken Sperm

O- and N-linked glycans were removed from the IPVL taken from laid chicken eggs by successive β-elimination and alkaline cleavage as described in Section 2.8.2. Following gel filtration, the orcinol sugar assay (see Section 2.10 for method) detected sugar in fractions 11-13. No protein was detected in these samples as determined by the BCA protein assay (see Section 2.11 for method). These fractions were pooled and found to contain 32.05 μg ml^{-1} of total sugars. Following de-salting the fractions containing sugars were again pooled and the sugar containing solution lyophilised and stored at -20°C

The mixture of O- and N-linked glycans was re-dissolved in 5 ml of NaCl-TES and a 1:10 dilution of the glycan solution was prepared in NaCl-TES and incubated with 3 separate samples of chicken spermatozoa at 30°C for 5 min (see Section 2.7.4 for method). Following incubation, the glycan-containing fraction was found to induce the AR in chicken spermatozoa. A significant increase was seen in the percentage of FITC-PNA labelled acrosomes in spermatozoa treated with the mixed glycan solution compared with control samples incubated in NaCl-TES alone (2-sample unpaired T-test; $P < 0.005$) (Figure 40).

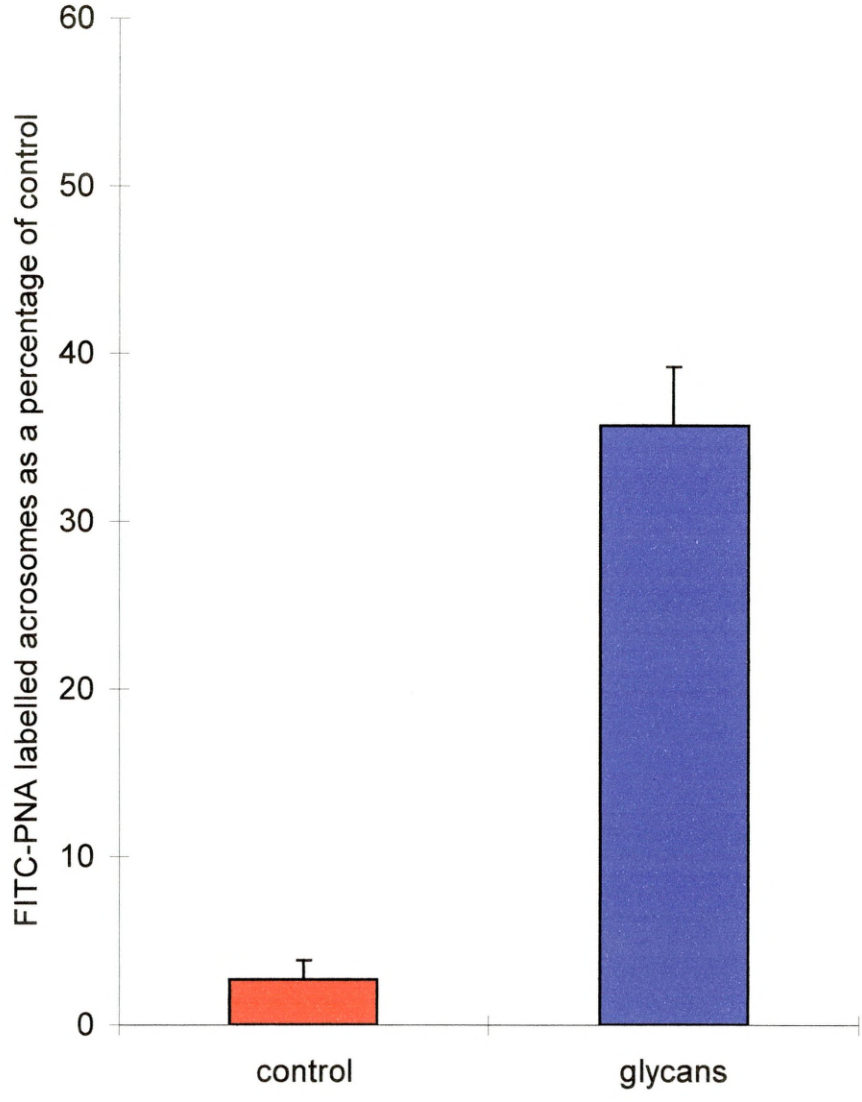


Figure 40. The effect of mixed glycans (containing both O- and N-linked glycans) removed from the IPVL of laid chicken eggs by acid hydrolysis on induction of the acrosome reaction in chicken spermatozoa. Glycans were obtained from a single experiment and used to induce the acrosome reaction in 3 separate sperm samples.

CHAPTER 4 DISCUSSION

4. DISCUSSION

4.1 CHARACTERIZATION OF AN *IN VITRO* SPERM-EGG INTERACTION ASSAY USING IPVL FROM LAID CHICKEN EGGS

An important prerequisite for studying the mechanisms involved in sperm-egg interaction in birds, is the development of suitable *in vitro* assay systems. In mammals, the advent of *in vitro* sperm-zonae interaction assays have proved valuable for studying the mechanisms involved in both the binding of spermatozoa to the zona pellucida and initiation of the AR. These assays have also found application in estimating the quality and therefore, potentially, the fertilizing ability of spermatozoa (Franken *et al.*, 1991; Gamzu *et al.*, 1992).

In avian species, equivalent *in vitro* assays for studying the mechanisms involved in the interaction of chicken spermatozoa with the IPVL have been developed using fragments from ovulated (Koyanagi *et al.*, 1988; Howarth, 1992) or follicular ova (Steele *et al.*, 1994), but have not been well characterised. They involve sacrifice of the hen and are therefore limited by the availability of material, as only one ovulated ovum and a limited amount of follicles can be obtained per bird.

The IPVL from laid chicken eggs was therefore investigated as a readily available and plentiful source of material for the *in vitro*

investigation of sperm-egg interaction in birds. Although Kido and Doi (1988) showed it was possible to separate the inner and outer perivitelline layers from laid chicken eggs by acid hydrolysis and demonstrated that the layers were structurally and chemically similar to the intact layer, their functional status was not assessed. Furthermore, it was not known if the acid treatment or the addition of the outer layer would affect the ability of the IPVL to be hydrolysed by spermatozoa. In fact Steele *et al.* (1994) suggested that the addition of the OPVL might induce conformational changes in IPVL proteins thus affecting the ability of sperm to hydrolyse the layer.

Examination of the protein profiles obtained following SDS-PAGE, for ovulated, follicular and laid perivitelline layers showed that the profiles obtained from both ovulated and follicular IPVL were similar, with 2 major bands at 78 kDa and 39 kDa. However, the staining intensity of the 2 major bands was consistently less for follicular IPVL (see Figure 10). This may be due to a difference in thickness of the IPVL samples, as proteins are still being synthesised in the developing follicles. The 78 kDa band was also present in the inner and whole perivitelline layer from laid eggs, but the 39 kDa band showed greater mobility at 35 kDa. This shift cannot be the result of acid hydrolysis as this band was also present in the whole perivitelline layer which was not subjected to this treatment. Steele *et al.* (1994) also demonstrated a shift in electrophoretic mobility of this protein in ovulated compared with laid IPVL and suggested that this change may be the result of postovulatory changes in the structure of the layer, or a

conformational change induced by the laying down of the outer layer. More recently Waclawek *et al.* (1998) demonstrated that this protein underwent a two-step decrease in size from follicular to laid eggs and suggested that this size reduction was probably due to proteolytic clipping, occurring firstly at ovulation and again when the oocyte has passed the site of fertilization. However, in the work presented here, no difference was seen in the molecular weight of the 39 kDa band taken from follicular and ovulated ova, suggesting that major modification of this protein occurs after ovulation, possibly during the deposition of the OPVL.

Whatever the reason for the increased mobility of the 39 kDa IPVL protein in laid eggs, these changes are unlikely to provide a chemical block to the penetration of spermatozoa, since the separated inner layer still retains sperm-binding and activating functions (see Section 3.3.1). This suggests that the role of the outer layer is to act as a mechanical barrier to the further penetration of spermatozoa as previously suggested (Howarth and Digby, 1973). Other electophoretic studies have demonstrated major PVL peptides of different molecular weights: 32 kDa, 183 kDa and 1000 kDa (Kido and Doi, 1988); and 33 kDa, 54 kDa and 200 kDa (Howarth, 1992). However, both these studies utilised non-reducing SDS-PAGE and Howarth (1992) used IPVL which was first solubilised in 5 mmol l^{-1} phosphate buffer.

Although it has previously been shown that IPVL from ovarian follicles and from freshly-ovulated ova, were equally responsive to

hydrolysis by spermatozoa (Steele *et al.*, 1994) the likelihood that the IPVL from laid eggs would show a similar response to sperm hydrolysis could not have been predicted from the earlier studies of Kido and Doi (1988). In this work the response of IPVL from laid eggs to hydrolysis by spermatozoa was investigated and compared with the response of IPVL from ovulated and follicular ova. Each successful sperm-IPVL event was identified as a small 'hole' in the layer.

Isolated fragments of IPVL from ovulated, follicular and laid eggs were taken from the same bird to minimise variability and were found to have a similar response to hydrolysis by spermatozoa in terms of the number of points of hydrolysis produced during a 5 min assay at 40°C (see Figure 11). This shows that neither the acid hydrolysis nor the laying down of the outer layer affects the activity of the sperm binding function of the IPVL.

Variability in the response of IPVL from the same and different laid eggs to hydrolysis by spermatozoa was investigated. Pieces of IPVL taken from the same laid egg were found to have a similar ability to be hydrolysed by the same semen sample (see Section 3.3.2). However, the number of points of hydrolysis was found to be significantly different when the IPVL fragments were taken from different eggs. Thus the source of IPVL is an important experimental variable and in order to minimise variability due to IPVL quality, IPVL fragments taken from the same laid egg should be considered an 'experimental unit'. A similar situation has

also been documented with mammalian systems where human hemizona assays showed up to 14% variability in spermatozoa binding capability in one ZP, but over 100% variation between different zonae (Franken *et al.*, 1991). In mammalian systems only two identical pieces of zona can be used as an 'experimental unit' whereas in the avian system, it is possible to obtain up to 20 replicate pieces of IPVL from the same laid egg which can be used in each experiment.

Another important experimental variable is the source and quality of the semen sample. Semen from 8 individual male birds were assessed for their ability to form points of hydrolysis in IPVL fragments from the same laid egg. Each male was found to have a different and characteristic ability to hydrolyse IPVL fragments from the same laid egg and a significant difference was seen in the number of points of hydrolysis produced in the IPVL by the different male birds tested (see 3.3.3). These results correspond with previous findings that individual male fowl differ considerably in their sperm quality and therefore fertilizing ability (Wishart and Palmer, 1986; Chaudhuri *et al.*, 1988).

The frequency of IPVL-holes was found to increase linearly with the concentration of spermatozoa used in the assay. At higher sperm concentrations, detection and counting of individual holes became inaccurate because, as for prolonged incubations, single holes began to coalesce, or the IPVL disintegrated. Bramwell and Howarth (1992b) reported a similar effect with IPVL from freshly-ovulated ova, but they also

found that at concentrations above 2 x 10^6 spermatozoa ml^{-1} this relationship was asymptotic. However, this reduction in the number of identifiable holes is likely to be due to single holes merging, thus reducing the number of IPVL-holes seen.

Hydrolysis of the IPVL was found to have occurred within 2.5 min of incubation with only the size of the holes increasing with further incubation as the proteolytic enzymes, released after induction of the AR, continued to digest the layer. After 5 min the number of holes had not increased significantly but were much clearer, so 5 min was chosen as the optimal incubation time for the assay. Prolonged incubation times resulted in large IPVL-holes that tended to coalesce, making counts inaccurate (see Figure 16).

The IPVL separated from laid eggs was therefore found to be a suitable substrate for the *in vitro* study of sperm-egg interaction in chickens. It was found to have a similar response to hydrolysis by spermatozoa as the IPVL from ovulated and follicular ova, but had the advantage of providing a large and readily available source of material that did not require sacrifice of the bird. Standard assays employed incubating pieces of IPVL with 1.25 x10^7 sperm ml^{-1} in DMEM at 40°C for 5 min and the assay was quantified by counting the number of points of hydrolysis produced in the IPVL. The source of IPVL and semen are important experimental variables and the use of IPVL from the same laid egg provides an internally controlled bioassay. It was possible to obtain

approximately 20 replicate pieces of IPVL from a single egg, which could be used as an 'experimental unit', providing a good method for studying and manipulating sperm-IPVL interactions *in vitro*.

4.2 APPLICATION OF THE *IN VITRO* SPERM-IPVL INTERACTION ASSAY FOR ASSESSING THE FERTILISING ABILITY OF SEMEN

4.2.1 Evaluation of Sperm Quality in Fresh Ejaculates of Semen from Individual Male Domestic Fowl by Assessment of Sperm-IPVL Interaction *In Vitro* and *In Vivo*

Determination of sperm quality is important in the poultry industry where it would be of considerable economic benefit to the breeder, to be able to select male birds on the basis of their potential fertilizing ability. Previous work using standard sperm quality assays (Wishart and Palmer, 1986; Chaudhuri *et al.*, 1988) has shown that individual male fowls differ in sperm quality and that this is reflected in their fertilizing ability. Although several *in vitro* assays for assessing sperm quality are currently available, and the results of some of these tests have been shown to be highly correlated with the fertilizing ability of freshly ejaculated semen, they have not been widely applied. One of the major criticisms of these tests is that they only assess a single parameter, such as motility or metabolic activity and are therefore unlikely to be sufficient to mirror the multifunctional

process of fertility. The *in vitro* sperm-IPVL assay utilising IPVL from laid eggs was therefore applied as a quantitative test of sperm quality which requires that the spermatozoa are motile, able to bind to and hydrolyse the IPVL, undergo the AR and finally, penetrate the layer.

Sperm samples from 8 individual male fowls, were found to have a different and characteristic ability to hydrolyse the IPVL in the *in vitro* sperm-IPVL assay (see 3.3.3). The results of the assay, for each of these males, were linearly correlated with sperm ATP (see 3.4.1.1) and motility (Robertson *et al.*, 1998) which in turn, have been shown to correlate with fertility in hens inseminated with low (approximately 10 x 10^6) doses of spermatozoa (Wishart and Palmer, 1986; Chaudhuri *et al.*, 1988). Therefore the argument that single function tests are inferior to multifunctional tests, such as the sperm-IPVL assay, are unfounded for fresh semen samples. However, the major advantage of the sperm-IPVL assay is that it is relatively unsophisticated, requiring only a simple low powered microscope.

The number of holes formed in the IPVL in the *in vitro* assay correlated logarithmically with the percentage of fertile eggs laid by hens inseminated with normal (60 x 10^6) doses of spermatozoa and linearly with the number of IPVL-holes formed *in vivo*, at areas away from the GD region (see 3.4.1.2). The relationship between insemination dose and fertility is non-linear and complex (Bramwell *et al.*, 1995; Wishart, 1985), so that often a large increase in the numbers, or quality of inseminated

spermatozoa will show only a small difference in the number of fertile eggs laid (Wishart, 1989). Therefore, the relationship between IPVL-holes formed in the *in vitro* assay and those formed *in vivo* at areas away from the GD was assessed. These holes have been shown to be linearly correlated with the numbers of spermatozoa inseminated (Wishart and Wood, 1994; See Wishart, 1995) and are representative of the population that have survived the female tract and ultimately interact with and hydrolyse the IPVL. The results of the *in vitro* sperm-IPVL assay were linearly correlated with the number of functionally active spermatozoa transferred to the egg at fertilization, which in turn correlates with fertility. It is evident therefore, at least for fresh sperm samples, that the characteristics which enable spermatozoa to bind and hydrolyse the IPVL *in vitro*, appear to be related to those that are limiting for sperm transport and storage within the oviduct, although the precise mechanisms involved remain unknown.

Whilst breeding efficiency is normally measured as fertility, it is fundamentally the result of successful sperm transfer. The results of the *in-vitro* sperm-IPVL assay were linearly correlated with the results of standard sperm quality assays (see 3.4.1.1; Robertson *et al.*, 1998) and with the number of functionally active spermatozoa transferred to the egg at fertilization, which in turn correlates with fertility (see 3.4.1.2). The *in vitro* sperm-IPVL assay therefore, provides a reliable and quantitative measure of sperm quality but has the advantage of being a more robust and less complex measurement with which to assess breeding efficiency.

4.2.2 Application of the *In Vitro* Sperm-IPVL Assay in Determining the Fertilizing Ability of Poultry Semen Stored At 5°C

Semen dilution and storage is important in the poultry industry in the development of AI techniques. Application of AI technology is essential for breeding modern strains of turkeys, as the broad-breasted turkey is incapable of breeding naturally (see Bakst and Brillard, 1995; Lake, 1995). In the chicken industry natural mating is still the method of choice for the production of laying stocks as the rate of fertilization cannot be surpassed by current AI methods (see Etches, 1996). Therefore, application of AI technology is currently limited to primary breeders, for the production of genetically superior males. However, this situation may soon have to be reviewed, as the traits that are economically desirable to the industry, such as rapid growth rate, are negatively correlated with successful reproduction.

The widespread use of AI technology has been inhibited by the rapid deterioration of the fertilizing ability of semen that has been stored, either as a liquid or cryopreserved (Wishart, 1989; see Wishart, 1995). One of the major limiting factors has been the lack of adequate *in vitro* tests for predicting the fertilizing ability of stored semen as current *in vitro* sperm quality assays, although adequate for predicting the fertilizing ability of fresh semen (Wishart and Palmer, 1986; Chaudhuri and Wishart, 1988), tend to grossly overestimate the fertilizing ability of semen that has been stored (Wishart, 1989). It has been proposed that during storage,

particularly at 5°C, that sperm undergo some subtle damage that is not detected by single function tests (Fulcher *et al.*, 1988; see See Wishart, 1995). The *in vitro* sperm-IPVL assay (see Section 2.6.3 for method) was therefore applied as a multifunctional test of sperm quality, which requires that sperm retain the range of functions required of them *in vivo* to hydrolyse the IPVL.

After storage at 5°C for 24 h, under aerobic and anaerobic conditions, both the dye exclusion test and the tetrazolium dye reduction assay estimated that at least 80% of chicken spermatozoa were viable and functionally intact (see 3.4.2.1). The *in vitro* sperm-IPVL assay however, showed a vast reduction in the ability of chicken spermatozoa to hydrolyse the IPVL (to approximately 52% for aerobic and 95% for anaerobic) and was the only assay to detect a difference between the two methods of storage. Although chicken spermatozoa are known to have a high glycolytic capacity, aeration of semen samples during low temperature liquid storage has previously been shown to improve the maintenance of sperm fertilizing ability (Wishart, 1981). However, no good quantitative assessment of the effects of liquid storage on fertilizing ability has been carried out, as it has for cryopreserved spermatozoa (Wishart, 1985) and the *in vitro* sperm-IPVL assay may highlight the true nature of the effect of storage of chicken semen at 5°C.

Turkey spermatozoa by comparison, have a lower glycolytic capacity and an absolute requirement for the presence of oxygen for

optimal ATP production (Wishart, 1982). A significant difference was seen in sperm quality between fresh and stored samples in all tests, but the eosin dye exclusion assay failed to detect any significant difference between the 2 methods of storage. Although the tetrazolium dye reduction assay, showed a difference in sperm quality between the 2 storage methods (see 3.4.2.2), this was only reduced to 65% in anaerobically stored samples compared with 82% for aerobically stored samples. The sperm-IPVL assay however, show a marked difference between fresh and stored spermatozoa samples where the ability to hydrolyse the layer was reduced to about 30% for spermatozoa stored aerobically, while this activity was virtually lost in samples stored under anaerobic conditions. The results of these experiments indicate that subtle changes occurred during storage which could not readily be detected by the dye exclusion or dye reduction tests and that these changes may involve alterations to the sperm surface, which affect the ability of spermatozoa to bind and hydrolyse the IPVL.

The sperm-IPVL assay can also be used to reflect the fertilizing ability of spermatozoa samples following cryopreservation. Extensive fertility trials have shown that less than 2% of cryopreserved chicken spermatozoa retain their fertilizing ability (Wishart, 1985). However, most current *in vitro* tests of sperm quality overestimate the fertilizing ability of cryopreserved chicken spermatozoa, indicating that 30-40% are metabolically and functionally intact (see Wishart, 1995). Robertson *et al.* (1997a) also found that the tetrazolium dye reduction assay estimated that

approximately 30% of spermatozoa were viable, and therefore potentially able to fertilize the ovum. In comparison the *in vitro* sperm-IPVL assay showed that only 8.5% of spermatozoa retained the ability to hydrolyse the layer, which is much closer to the predicted rate of fertilization. Cramer *et al.* (1994) also observed a reduction in the fertilizing ability of cryopreserved chicken spermatozoa and related this to a reduction in the ability of spermatozoa to bind to the ovum. They found that after cryopreservation the number of spermatozoa capable of binding to immobilised proteins from the whole PVL (i.e. the inner and outer perivitelline layers) was reduced to 18%. However, they also found that spermatozoa from several mammalian species bound to these proteins, whereas in this work spermatozoa from bull, rat, and mouse were unable to hydrolyse the IPVL separated from laid chicken eggs (see Section 3.5.3) Therefore it appears that the method of Cramer *et al.* (1994) appears to be assessing a different parameter from the assay described here.

Most current avian *in vitro* tests of sperm quality are limited in the parameters which they measure, compared to the many functions required of spermatozoa for successful fertilization after intravaginal insemination (see Bakst *et al.*, 1994). It has been proposed that one major fault of these tests is that none assess characteristics of the sperm surface, which are clearly important for both migration of the sperm through the vagina (Steele and Wishart, 1992a) and binding to the IPVL, and appear to be compromised during liquid semen storage (Fulcher *et*

al., 1988). The sperm:IPVL interaction assay requires that spermatozoa are able to carry out a range of functions that are required of them *in vivo* to fertilize the ovum, including - motility, binding to the IPVL, induction of the AR and hydrolysis of the IPVL. Each of these events requires a complex interaction of sperm metabolic and regulatory parameters. Therefore, the sperm-IPVL test is likely to be highly discriminatory against compromised spermatozoa.

4.3 SPECIES SPECIFICITY OF SPERM-IPVL INTERACTION

The importance of the IPVL in species-specific interaction of gametes in avian fertilization was investigated by determining the ability of chicken spermatozoa to hydrolyse IPVL from chicken, turkey, pheasant, peahen (*Galliformes)*, duck *(Anseriformes)* and zebra finch (*Passeriformes*) laid eggs in the *in vitro* sperm-IPVL assay (see 2.5.4 for method). Chicken spermatozoa were found to hydrolyse the IPVL from all species assessed (see Table 1) indicating that sperm-IPVL recognition and binding in aves is not entirely species-specific and that some degree of cross-reactivity occurs between the species.

It is generally believed that heterologous fertilization occurs more readily between closely related species (Roland *et al.,* 1985) and in agreement with this, chicken spermatozoa were found to preferentially hydrolyse IPVL from the order *Galliformes*, although variations were found

between and within the different subfamilies. Within this order, turkeys (family *phasianidae*, subfamily *meleagrinae*) are more distantly related to chickens than pheasant, or peahen (both family *phasianidae*, subfamily *phasianinae*) (Pycraft, 1931). However, chicken spermatozoa interacted more readily with the inner layer from turkey eggs, than that of homologous IPVL, indicating no species-specificity at the level of sperm-IPVL interaction between these two birds (see Table 1). Hydrolysis of the IPVL varied within the subfamily *phasianinae*, with chicken spermatozoa hydrolysing pheasant IPVL almost as well as homologous IPVL (91%) but only able to hydrolyse peahen IPVL with 64% efficiency. It would appear therefore that species-specificity at the level of sperm-IPVL interaction may not be directly related to species divergence within the order *Galliformes*.

Cross-reactivity was also seen following incubation of chicken spermatozoa with IPVL from duck and zebra finch eggs, although to a much lesser degree (see Table 1). This may have been expected as these birds belong to different avian orders from the chicken (*Anseriformes* and *Passeriformes* respectfully).

Bramwell and Howarth (1992c) also reported limited species-specificity at the level of sperm-IPVL interaction within avian species. They showed that pre-treatment of chicken, turkey or duck spermatozoa with solubilised preparations of IPVL for 20 min, in homologous and heterologous combinations inhibited the interaction of pre-treated

spermatozoa with subsequent intact fragments of homologous IPVL *in vitro*. However, chicken spermatozoa incubated with homogenised preparations of IPVL for 5 min show a loss of metabolic activity as assessed by the tetrazolium dye reduction assay (see Section 3.7.1.3) and also showed an increased permeability to eosin dye (see Section 3.7.2.2.1), suggesting that chicken spermatozoa may have a limited life span following induction of the AR. Also pre-incubation of spermatozoa with IPVL would result in a population of spermatozoa that had acrosome reacted, therefore may be unable to bind to the IPVL.

No obvious relationship was seen in the ability of chicken spermatozoa to hydrolyse IPVL from the different avian species and the electrophoretic profiles obtained by SDS-PAGE. In each of the species analysed, 2 major protein bands were found, with a 78 kDa band present in all samples, (see Figure 21). In chicken IPVL a second major band at 35 kDa was present as before (see Figure 10) but was absent in turkey IPVL although it was preferentially hydrolysed by chicken spermatozoa. Turkey IPVL showed a second major band at 39 kDa band. The 39 kDa band was also present in peahen IPVL, although hydrolysis of this layer by chicken spermatozoa was reduced to 64% of the control (chicken spermatozoa-chicken IPVL interaction). Duck and zebra finch IPVL, which were poorly hydrolysed by chicken spermatozoa, showed a second major protein band at 41 kDa.

Several lines of evidence currently suggest that sperm recognise and bind to specific oligosaccharides present on the ZP (Florman and Wassarman, 1985; Bliel and Wassarman; 1988, see Wassarman and Litscher, 1995) and that these sugars may play an important role in the species-specific recognition and binding of mammalian gametes (Skutelsky *et al.*, 1994). Carbohydrates have also been shown to be an important determinant in sperm-IPVL interaction in avian systems, (Howarth, 1992) and it may be that specific sugar residues are important in the species-specific recognition and hydrolysis of IPVL proteins by spermatozoa.

Although cross-reactivity of sperm-IPVL interaction was seen for all species assessed, no interaction was seen between chicken IPVL and mammalian spermatozoa. In mammals, uncapacitated spermatozoa will often attach to the zona pellucida of heterologous species in a non-specific manner (see Bedford, 1981; Fournier-Delpech and Courot, 1987), but uncapacitated spermatozoa from rat, mouse and bull failed to form any attachment to the chicken IPVL in the *in vitro* sperm-IPVL assay (see 3.5.3). Capacitation results in surfaces changes which confers on the spermatozoon, the ability to bind specifically to the zona pellucida, undergo the AR and ultimately penetrate the egg investments. However, capacitated mammalian spermatozoa also failed to bind to, or form points of hydrolysis in the chicken IPVL. These results appear contrary to those of Barbato *et al.* (1998) who found that spermatozoa from several mammalian species, including rat, mouse and bull, bound to immobilised

proteins from the whole perivitelline layer (i.e. containing both inner and outer perivitelline layer proteins). However, it appears that the method of Barbato *et al.* (1998) may be assessing a different parameter and perhaps sperm binding was non-specific, to protein(s) present within the whole perivitelline layer.

From the results obtained, it appears that incomplete species-specificity occurs at the level of sperm-egg interaction in birds, but no cross-reactivity occurs between avian and mammalian species. The limited interaction of chicken spermatozoa with duck *(Anseriformes)* and zebra finch (*Passerifirmes*) IPVL suggests an order dependent specificity at the level of sperm-egg interaction in birds. However, the extent of sperm-IPVL interaction may not be dependent on the degree of relatedness within an order, as indicated by the results obtained within the order *Galliformes*.

4.4 NATURE OF THE PREFERENTIAL HYDROLYSIS OF THE IPVL OVERLYING THE GERMINAL DISC

The large megalecithal ovum of the bird poses a particular problem to spermatozoa as, in order for fertilization to occur, they must penetrate a very small area, the GD region at the animal pole, on the surface of a very large yolk mass (see Figure 4). It seems conceivable therefore, that a mechanism should exist to ensure that spermatozoa preferentially

penetrate the IPVL overlying this region, which houses the female genetic material and organelles (Bakst and Howarth 1977a; Perry *et al.*, 1978).

Previous *in vitro* studies have demonstrated that chicken spermatozoa preferentially hydrolyse the IPVL overlying the GD region in the intact ovum, although these results were not quantitated (Howarth and Digby, 1973; Ho and Meizel, 1975; Bakst and Howarth, 1977b). Later studies showed that in oviposited eggs, points of hydrolysis were found to be concentrated up to 20 fold in the IPVL overlying the GD region (Bramwell and Howarth, 1992a; Steele *et al.*, 1994; Wishart, 1997) at the animal pole. However, holes are also found in the IPVL from other regions of the ovum, but to a much lesser degree and it is currently not known whether these are produced at the time of fertilization or, as suggested by Steele *et al.* (1994) are artefacts resulting from digestion by the hydrolytic enzymes of disintegrating spermatozoa, trapped during the formation of the egg.

The reason for the increased hydrolytic activity at the GD is unknown, but several hypothesis have been proposed. Howarth and Digby (1973) found that spermatozoa caused preferential rupture of the IPVL overlying the GD following a 30 min incubation *in vitro* and suggested chemotactic attraction of spermatozoa to this region. Ho and Meizel (1975) questioned this, as they found that partially purified extracts of cock acrosin were also able to cause preferential hydrolysis of the IPVL overlying the GD and suggested that the IPVL overlying this region was

thinner than elsewhere on the ovum. Bakst and Howarth (1977b), suggested that factors that may activate sperm acrosomal activity, such as Ca^{2+} or Mg^{2+} may only be available at the animal pole. However, this is unlikely as these ions are present in the medium used for IVF (Howarth and Digby, 1973) and Ca^{2+} has been shown to be present in the fluid of the ovarian pocket at the time of ovulation *in vivo* (Ashizawa and Wishart, 1992). Bramwell and Howarth, (1992b) proposed an increase in the concentration of sperm binding proteins at the animal pole as a possible mechanism for preferential hydrolysis of the IPVL at this region. The GD underlying the IPVL at the animal pole has been shown to be quite distinct from other regions of the ovum (Perry *et al.*, 1978). Bakst (1978) found that the microvillar projections of the oolemma were longer and more abundant at the animal pole and proposed that they may in fact be the site of sperm binding proteins. However, later Bakst (1979) postulated that these projections may be remnants of granulosa cell microvilli of the preovulatory oocyte.

In this study, initial experiments involving *in vitro* fertilization of the intact chicken ovum, support the hypothesis that chicken spermatozoa preferentially hydrolyse the IPVL overlying the GD (see Section 3.6.1). However, when fragments of non-GD IPVL were removed and further incubated *in vitro* under the same conditions as the intact ovum (see Section 2.6.2), the number of IPVL-holes increased to numbers comparable to those found over the GD region after *in vitro* fertilization (see Figure 22). Therefore, it appears that sperm binding proteins are

evenly distributed throughout the IPVL. This is supported by the work of Steele *et al.* (1994) who failed to show preferential hydrolysis of isolated fragments of IPVL taken from both the GD and non-GD regions in *in vitro* assays. Furthermore, they demonstrated that the peptide profiles of the IPVL overlying the GD and non-GD IPVL were indistinguishable after SDS-PAGE. These results are in contrast to those of Bramwell and Howarth (1992b), who reported an increased concentration of holes in isolated fragments of IPVL taken from over the GD, compared with IPVL from other areas. However, their conclusion was based on a single result in which the concentration of holes in the IPVL overlying the GD was only 1.33 times greater than those found in other areas.

Steele *et al.* (1994) suggested that the factor(s) responsible for the preferential hydrolysis of the IPVL overlying the GD region of the ovum may not be associated with the IPVL itself. One possible explanation may be that the yolk material has an inhibitory effect on hydrolysis of the IPVL at areas away from the animal pole, either acting as a mechanical barrier to the entry of spermatozoa, or due to some inhibitory factor present in the yolk, perhaps inhibiting the activity of acrosin. However, the addition of 10% egg yolk to the sperm-IPVL assay failed to inhibit hydrolysis of the IPVL, perhaps indicating that the concentration of any potential inhibitory factor(s) was too low, or absent. However, at concentrations greater than this sperm motility was inhibited as the solution became too viscous. Conversely in human spermatozoa the addition of egg yolk buffer has been found to increase the binding of spermatozoa to the zona pellucida

(Gamzu *et al.*, 1994) and decrease the frequency of spontaneous ARs during capacitation (Tesarik and Mendoza, 1995).

The incubation of spermatozoa with fragments of IPVL, excised from the ovulated ovum with approximately 5 mm yolk still attached, resulted in inhibition of IPVL hydrolysis to approximately 10% of control samples. Therefore, instead of spermatozoa being selectively attracted to, or more hydrolytically active towards the IPVL overlying the GD, it appears that spermatozoa are in fact inhibited from hydrolysing the IPVL away from the GD by the presence of yolk material. It is known that the plasma membrane of the ovum is only intact over the GD region (Bakst, 1978) and at areas away from this region, the discontinuous plasma membrane may allow the yolk material to have direct contact with and infiltrate the IPVL. Whether the inhibition of hydrolysis is due to a mechanical barrier caused by the penetrating yolk, or to some factor(s) present in the yolk, which is perhaps concentrated at the surface of the IPVL is presently unclear.

Microscopic analysis of the inner surface of the IPVL, which is in contact with the yolk, shows that it has a different morphology from the outer surface, which spermatozoa interact with in the intact ovum. The inner surface has an open, fibrous appearance, whereas the outer surface has an more closed, granular type appearance (see Figure 24). When spermatozoa were given access to only one side of the IPVL *in vitro* (see 2.6.3.1 for protocol), more holes were seen in the (approximately 3-fold)

outer, granular side of the IPVL. This indicates that there is a higher concentration of sperm binding proteins on this surface, which is the side that spermatozoa interact with *in vivo.*

4.5 THE AVIAN ACROSOME REACTION

In most species studied to date, the AR has been shown to be a necessary prerequisite to fertilization. The development of suitable routine methods for determining the acrosomal status of the spermatozoon has been important in several mammalian species, including human, for elucidation of the mechanisms involved in the AR and determination of its role in the fertilization process.

In mammals several methods are currently available to monitor the AR and the choice of assay system is dependent on the size or morphology of the acrosome of the species under study. A number of investigators have explored the use of several staining techniques for visualising spermatozoa with small acrosomes at the level of the light microscope, including FITC-labelled lectins and CTC staining. Some investigators have however, successfully used phase-contrast or DIC microscopy to determine the acrosomal status of rodent spermatozoa which are known to have small acrosomes (see Cardullo and Florman, 1993). The chicken spermatozoon has a small acrosome that is not easily identified by simple light microscopy, therefore the above methods were

applied to determine the acrosomal status of chicken spermatozoa, using IPVL to stimulate the physiological acrosome reaction.

4.5.1 Determination of a Suitable Routine Method for Evaluating the Acrosomal Status of Chicken Spermatozoa

Of the methods used to assess the acrosomal status of chicken spermatozoa, only the fluorescent labelled lectin PNA was able to reliably differentiate between acrosome-intact and acrosome-reacted spermatozoa. Other methods used were either unable to differentiate between exposed and intact acrosomes, or the results were equivocal.

4.5.1.1 Simple light microscopy

In this study, phase contrast, and DIC microscopy proved to be ineffective for determining the acrosomal status of chicken spermatozoa. These assays, although rapid and convenient, are only suitable for species possessing large acrosomes. The acrosomes of chicken spermatozoa were too small and similar in optical properties to the rest of the sperm to reliably detect changes in acrosomal status at x1000 magnification using simple light microscopy. In contrast to this, Koyanagi *et al.* (1988) used phase microscopy at x1000 magnification to detect 'acrosome-reacted' chicken spermatozoa. Following incubation of

spermatozoa with IPVL for up to 120 mins, a maximum of 26% spermatozoa were identified as 'acrosome-reacted' by this group, i.e. those that had a shorter, thinner acrosomal tip than normal 'acrosome-intact' spermatozoa. However, in the *in vitro* sperm-IPVL assay the formation of points of hydrolysis is completed in less than 5 mins (See section 3.3.5; Robertson *et al.*, 1997a). Therefore, the extended incubations carried out by this group may have resulted in dead and disintegrating spermatozoa that were lacking acrosomes, leaving only the perforatorium visible. These may have been the population of spermatozoa that were identified as 'acrosome-reacted' by this group.

4.5.1.2 Chlortetracycline staining

Fluorescent probes have been used successfully to determine the acrosomal status of mammalian spermatozoa (see Cross and Meizel, 1989). One such probe is CTC, a fluorescent antibiotic that has been used to monitor both capacitation and the AR in spermatozoa of several mammalian species, including mouse (Ward and Storey, 1984), human (DasGupta *et al.*, 1993) and bull (Fraser *et al.*, 1995). In this study bull spermatozoa were used as a positive control to test the efficacy of the CTC assay in detecting acrosome-reacted chicken spermatozoa, therefore only the 'F' and 'AR' patterns were scored (see Section 3.7.1.2.1). Following incubation of uncapacitated, freshly thawed, bull spermatozoa with CTC, less than 50% of sperm cells displayed the 'F' pattern. This was

not unexpected as freeze-thaw is likely to damage sperm cells causing loss of viability and membrane damage. However, following capacitation and induction of the AR with A23187, the percentage of fluorescent acrosomes decreased significantly, showing that the CTC assay was reliably detecting acrosome-reacted bull spermatozoa.

In chickens, the pattern obtained following CTC staining was the same for both acrosome-intact and acrosome-reacted spermatozoa. Intense fluorescence was seen over the mitochondria in all spermatozoa visualised and as a small spot where the acrosome meets the nucleus in more than 80% of spermatozoa (see Figure 26), regardless of their acrosomal status. It was difficult to determine if the staining at the base of the acrosome was present in all spermatozoa, as the fluorescence appeared to be restricted to one side and its presence may have been obscured due to the orientation of the sperm cell on the slide. Therefore this method was not suitable for detecting acrosome-reacted chicken spermatozoa.

4.5.1.3 Tetrazolium dye reduction assay

The tetrazolium dye reduction assay can be used to determine the metabolic activity of chicken sperm cells (see 3.4.2.1). Viable cells reduce the colourless INT salt to a highly coloured formazan, which is insoluble, and can be visualised at localised points within the cell. The location of

the area of metabolic activity at the base of the acrosome is similar to that found with CTC staining, with staining at the mitochondria and at the base of the acrosome (see Figure 27), although currently the function of this region is not known.

Following induction of the AR with homogenised preparations of IPVL, the colour intensity and the number of spots at both the base of the acrosome and at the mitochondria decreased, indicating loss of metabolic activity. Furthermore, assessment of sperm plasma membrane integrity, following aniline-eosin staining, shows that more than 50% of spermatozoa incubated with the IPVL were permeable to eosin dye (see Figure 31). Therefore, it may be that chicken spermatozoa have a limited life span following induction of the AR.

4.5.1.4 Lectin labelling

Previous studies have demonstrated that fluorescent labelled lectins are useful tools for determining the acrosomal status of spermatozoa in other animal groups. Several lectins have proved valuable due to their affinity for the acrosomal region of spermatozoa and their ability to differentiate between acrosome-intact and acrosome-reacted sperm. In this study, a range of lectins that have been used previously to determine the acrosomal status of spermatozoa in other

animals were tested for their ability to differentiate between unfixed, acrosome-intact and acrosome-reacted chicken spermatozoa.

Of the lectins used, only FITC-PNA was able to reliably differentiate between acrosome-intact and acrosome-reacted chicken spermatozoa. No detectable difference was seen in the staining patterns obtained between acrosome-intact and acrosome-reacted chicken spermatozoa with WGA, Con A, PSA, or TPA (see Appendix D for lectin specificities).

A uniform, intense staining of the whole spermatozoa was seen in acrosome-intact chicken spermatozoa following incubation with WGA. Spermatozoa were also extensively agglutinated, mainly at the flagellar region. No detectable change was seen in the staining or agglutination pattern following induction of the AR. However, it is possible that any changes that may have occurred in the acrosomal region following induction of the AR were not detected against the high staining intensity of the spermatozoa. Therefore this lectin was unsuitable for detection of the AR in chickens.

Con A was also unsuitable for determining the acrosomal status of chicken spermatozoa. It bound weakly to the acrosome and midpiece of both acrosome-intact and acrosome-reacted spermatozoa, showing the presence of ∝-man residues on both the plasma membrane and exposed acrosomes. Con A has been used to detect acrosome-reacted spermatozoa in several mammalian species, including human (Holden

and Trounson, 1991) and has been shown to preferentially bind to the IAM of acrosome-reacted sperm in this species.

PSA is one of the most extensively used probes for assessing the acrosomal status of mammalian spermatozoa and has been shown to bind to the acrosomal contents of fixed and permeabilised human spermatozoa (Cross *et al.*, 1986; Mendoza *et al.*, 1992). In chickens, FITC-PSA did not label unfixed, acrosome-intact chicken spermatozoa. Following induction of the AR with homogenised preparations of IPVL, only a small percentage (approximately 5%) of spermatozoa displayed a weak fluorescence over the acrosomal region. (see 3.7.1.4). It may be that the acrosomal contents of chicken spermatozoa lack ∝-man residues, or that the AR in these birds is completed so rapidly that the acrosomal contents are rapidly dispersed leaving only residual glycoproteins causing limited fluorescence.

The fucose binding lectin LTA showed extremely low levels of fluorescence along the entire length of the spermatozoa with no detectable difference between acrosome-intact or acrosome-reacted chicken spermatozoa. Fucose specific lectins, including LTA , have been used to detect acrosome-reacted human spermatozoa and has been shown to bind specifically to the equatorial segment of acrosome-reacted human spermatozoa (D'Cruz and Haas, 1996).

FITC-PNA was able to reliably detect acrosome-reacted chicken spermatozoa. An intense fluorescence was seen over the acrosome of more than 40% of spermatozoa that had been incubated with homogenised preparations of IPVL i.e. those had undergone the physiological AR, but largely absent in control samples that had been incubated under the same conditions without the addition of IPVL (see Section 3.7.1.4; Robertson and Wishart, 1996). Staining was exclusively limited to the acrosomal region of spermatozoa, making it a reliable method of measuring the AR in this species. Only a small percentage of FITC-PNA labelled acrosomes were found in control samples (typically less than 4%) and these are likely to be damaged spermatozoa that have previously exposed acrosomes. The majority of FITC-PNA labelled acrosomes remained attached to the spermatozoa, although a small percentage of acrosomal tips (typically less than 4 %), were detached from the spermatozoa and it was assumed that these were the result of mechanical damage. In fact Kerr (1997) used the FITC-PNA assay to show that following a 5 min incubation *in vitro*, acrosome-reacted chicken spermatozoa were able to pass through the IPVL with their acrosomes still intact.

Binding of FITC-PNA to acrosome-reacted spermatozoa indicates that the AR reveals previously hidden Gal(β-1-3)GalNAc residues on the exposed acrosomes of chicken spermatozoa. FITC-PNA has been shown to selectively label exposed acrosomes in toad spermatozoa (*Bufo japonicus*) (Takamune, 1987), and EM studies have shown that it binds

specifically to the IAM of this species. In chicken spermatozoa, electron micrographs indicate that PNA binds to the acrosomal matrix itself (see 3.7.3), therefore is able to reliably detect acrosome-reacted sperm.

FITC-PNA labelling of spermatozoa has previously been shown to be highly correlated with acrosomal status in several mammalian species, including boar (Fazeli *et al.*, 1997), human (Mortimer, 1987) and bull (Cross and Watson, 1994). In these species PNA has been shown to bind to the OAM of permeabilised spermatozoa and diminishes with the occurrence of the AR. The advantage of the avian system is that the population of spermatozoa that have undergone the AR are identified by an increase in fluorescence and prior fixation of spermatozoa is not required.

4.5.2 Non Physiological Methods of Exposing the Acrosomes of Chicken Spermatozoa

It was initially assumed that the calcium ionophore A23187 would induce the AR in chicken spermatozoa. In most species studied A23187, in the presence of extracellular Ca^{2+}, acts to increase the permeability of the plasma membrane to Ca^{2+} causing an increase in cytoplasmic free Ca^{2+} and acrosomal exocytosis (see Fraser, 1987). However, incubation of chicken spermatozoa with A23187, in the presence of extracellular Ca^{2+}, failed to induce the AR and incubation in this medium for as little as

5 min, resulted in a loss of sperm viability, as assessed by the tetrazolium dye reduction assay (see 3.7.1.3). It may be that the concentration of A23187 was too high for chicken spermatozoa, or that they require only a small amount of extracellular Ca^{2+} at physiological temperatures for acrosomal exocytosis and a large influx of this ion is detrimental to the cell, making this an unsuitable method for induction of the avian AR.

The detergent digitonin also proved to be unsuitable for exposing FITC-PNA binding sites on the acrosomes of chicken spermatozoa. At concentrations below 25 μg ml^{-1} digitonin, the plasma membrane of chicken spermatozoa largely remained intact and no significant FITC-PNA staining was seen over the acrosomes of treated spermatozoa, compared with control samples. Although digitonin concentrations of 25 μg ml^{-1} disrupted the plasma membrane in almost 50% of spermatozoa, the percentage of FITC-PNA labelled acrosomes did not increase significantly, indicating that the OAM remained intact, preventing binding of FITC-PNA to the acrosome. The greatest percentage of permeabilised plasma membranes were found in spermatozoa incubated with 50 μg ml^{-1} digitonin, but only a small increase to approximately 20% of FITC-PNA labelled acrosomes was found. However, this concentration of digitonin resulted in fragmentation of many of the spermatozoa and therefore was also deemed unsuitable for exposing the chicken acrosome.

Sonication of chicken spermatozoa for 1 s, resulted in disruption of the plasma membrane, as assessed by aniline-eosin smears (see Section

3.7.2.2.1), and an increase in PNA binding sites on the exposed acrosomes indicating that the OAM membrane was also disrupted (see Section 3.7.2.2.2). Increased sonication times resulted in fragmentation of the spermatozoa, many without acrosomes, therefore these samples were not processed further. Sonication has also been shown to expose PNA binding sites on the IAM of toad *Bufo japonicus* spermatozoa (Takamune, 1987).

An important feature of the eutherian mammalian acrosome is its fragility. Capacitated spermatozoa readily undergo spontaneous or induced acrosome reactions and this has led to the suggestion that capacitation may involve removal of acrosome stabilising agents which act to prevent premature AR (Bedford, 1983). In contrast marsupial spermatozoa have been shown to be resistant to disruption of the acrosome following exposure to A23187 and a range of other cell activating agents (Mate and Rodger, 1991). These animals appear to have unusually stable acrosomes which remain intact under a wide range of conditions that disrupt the acrosomes of placental mammals.

Chicken spermatozoa also appear to have stable acrosomes. They have a low level of degenerate AR (see Section 3.7.1.4) and the acrosome is not exposed following treatment with A23187 (see Section 3.7.2.1) or digitonin (see Section 3.7.2.2). Avian spermatozoa do not seem to undergo capacitation (see Section 1.4.1.2) and as they reside in the oviduct for extended periods (see Section 1.3.1) and it would seem

inappropriate for oviductal secretions to initiate the process of membrane destabilisation.

4.5.3 FITC-PNA Labelling of Acrosome-Reacted Chicken Spermatozoa Following Incubation with Solubilised Fractions of IPVL

IPVL proteins, were successfully solubilised in 5 mmol NaH_2PO_4 l^{-1} pH 2.5 (Bramwell and Howarth, 1992C; Howarth, 1992). Following separation of the proteins by FPLC, a single fraction of apparent MW of 42 kDa, corresponding to peak one was found to contain AR inducing activity in chicken spermatozoa. Approximately 50% of spermatozoa incubated, for 5 min at 30°C, with these fractions showed FITC-PNA labelling of exposed acrosomes, whereas fractions corresponding to peaks 2 - 6 did not show significantly increased FITC-PNA acrosomal staining above control samples (see Section 3.7.1.4.1). The AR assays had to be carried out immediately as the IPVL proteins, which have been shown to be hydrophobic in nature, (Kido *et al.*, 1975) quickly re-aggregated when the pH returned to pH 7.4. As a result the spermatozoa were found concentrated in the pieces of IPVL making counts inaccurate.

4.5.4 Species-Specificity of FITC-PNA Binding to Acrosome-Reacted Spermatozoa

Spermatozoa from chicken, turkey and quail displayed a similar ability to bind FITC-PNA following induction of the AR with homologous preparations of IPVL (see Section 3.7.5). As seen before with chicken spermatozoa (see Section 3.7.1.4), little or no staining was found in control, acrosome-intact spermatozoa that were incubated in DMEM alone. However, with each of the species assessed, this increased to approximately 40% in samples incubated with homologous IPVL i.e. acrosome-reacted spermatozoa. Therefore the AR must reveal, in spermatozoa from all of these species, previously hidden Gal(β-1-3)GalNAc residues on the exposed acrosomes of all of these species.

4.5.5 The Effect of Extracellular Ca^{2+} and Temperature on Induction of the Avian AR

Extracellular Ca^{2+} is known to be required for induction of the AR in most species studied to date (see Fraser, 1987). In agreement with this, the addition of extracellular Ca^{2+} was found to be an absolute requirement for acrosomal exocytosis in chicken spermatozoa at 40°C, the normal body temperature of these birds. When incubated with preparations of IPVL in a simple buffered salt solution at 40°C chicken spermatozoa were unable to undergo the AR, as determined by FITC-PNA labelling of

exposed acrosomes (see Section 3.7.4). However, the addition of 5 mmol l^{-1} $Ca^{2+}$to the salt solution resulted in a significant increase in acrosomal exocytosis. At 30°C, chicken spermatozoa were able to undergo acrosomal exocytosis, both in the presence, or absence, of extracellular Ca^{2+}, and the IPVL was found to be the sole activator of the AR at this temperature. The reason for the temperature dependent requirement for extracellular Ca^{2+} for induction of the AR is not known. However, this phenomenon is also found in the regulation of chicken sperm motility (Wishart and Ashizawa, 1987; Thomson and Wishart, 1989; 1991). When incubated in a simple salt-based buffer at 40°C, chicken spermatozoa are immotile, but if the temperature is lowered to 30°C, or Ca^{2+} is added to the medium, motility is restored (Wishart and Ashizawa, 1987; Ashizawa *et al.*, 1989). Interestingly this temperature-dependent requirement for extracellular Ca^{2+} for motility appears to be species dependent. Drake spermatozoa have been shown to display this temperature-dependency to Ca^{2+}, whereas turkey, quail and Houbara bustard spermatozoa do not (Wilson and Wishart 1996). It is currently not known if this extends to the AR in these species.

4.5.6 Proposed Mechanism of the Avian AR

There has been considerable controversy over the nature of the avian AR (see Section 1.4.4.2). Previously, Okamura and Nishiyama (1978a) proposed that the avian AR was more similar to that of

invertebrates than to mammals. They proposed that as spermatozoa contact the IPVL, the plasma and outer acrosomal membranes fuse, open at the apical region and the acrosomal contents are released, dissolving a 'tunnel' through the layer. 'Acrosome-reacted' spermatozoa with acrosomes consisting of only the perforatorium surrounded by the IAM enter the perivitelline space and fuse with the plasma membrane (see Figure 6).

In this study, visualisation of the binding of spermatozoa to the IPVL by phase microscopy indicates that chicken spermatozoa do not attach to the IPVL at the tip of the acrosome, but rather the acrosome appears to lie flat against the layer. Upon binding, the spermatozoa initially show a 'birling' motion, followed by a slower oscillating type of movement. Acrosomal proteases are then released, resulting in lysis of the IPVL and the formation of a 'hole'. The exact timing of the fowl AR is not known. However, it must be initiated within 2.5 min of binding to the IPVL when the 'holes' are first visible (see 3.3.5; Robertson *et al.*, 1997a), and is completed within 5 min when FITC-PNA binding sites are revealed on the exposed acrosomal matrix (see Section 3.7.1.4.; Robertson and Wishart, 1996). It was not possible to ascertain from the electron micrographs (L Holm, Swedish University of Agricultural Sciences, Uppsala, Sweden), whether fusion of the plasma and outer acrosomal membranes occurred, as no hybrid vesicles were found. However, spermatozoa incubated with homogenised preparations of IPVL for 5 min resulted in removal of both the plasma and outer acrosomal membranes and FITC-PNA labelling of

the acrosomal matrix was seen. Unlike the proposed mechanism of Okamura and Nishiyama (1978a), spermatozoa appear to penetrate the IPVL with their acrosomes still attached (see 3.7.1.4.1; Kerr, 1997).

4.6 THE ROLE OF CARBOHYDRATES IN SPERM-IPVL INTERACTION IN BIRDS

Considerable experimental evidence exists to support the view that gamete interaction in mammals (see Wassarman and Litscher, 1995; Tulsiani *et al.*, 1997; Shalgi and Raz, 1997) and at least some non-mammals including ascidian, (Rosati and De Santis, 1980) and sea urchin (Kinsey and Lennarz, 1981), is a carbohydrate mediated event. Competitive inhibition studies using various lectins, monosaccharides, polysaccharides or glycoproteins have indicated a role for specific carbohydrate moieties in mediating sperm-zona interactions in several mammalian species including hamster, human, guinea pig (Huang *et al.*, 1982); rat (Shalgi *et al.*, 1986) and mouse (Shur and Hall, 1982; Bleil and Wassarman, 1988; Cornwall *et al.*, 1991). Treatment of either the ZP or spermatozoa with glycosidases has also been used to demonstrate the importance of carbohydrates in sperm-egg binding (e.g. Shur and Hall, 1982; Florman and Wassarman, 1985; Shalgi *et al.*, 1986).

In birds, the specific molecules involved in sperm-IPVL interaction have not yet been identified but, as with the mammalian zona, the IPVL

has been shown to be composed of glycoproteins (Kido *et al.*, 1975; Kido and Doi, 1988). Preliminary experiments into the role of IPVL glycans in avian sperm-egg interaction demonstrated that pre-treatment of spermatozoa with solubilised IPVL prior to co-incubation with IPVL sheets significantly reduced hydrolysis of the layer (Howarth, 1990). Further work showed that removal of the carbohydrate portion of the IPVL prevented this inhibitory effect (Howarth, 1992). These results were taken to demonstrate that the glycan portion of the IPVL was essential for mediating sperm-IPVL interaction. However, pre-incubation of spermatozoa with IPVL extracts induces the AR within 5 min (Robertson and Wishart, 1996; Robertson *et al.*, 1997b) resulting in a population of spermatozoa with mixed acrosomal status. It is not known if acrosome-intact (as in mouse: Saling and Storey, 1979; Bleil *et al.*, 1988) or acrosome-reacted spermatozoa (as in guinea pig: Huang, *et al.*, 1981) bind to the IPVL in avian species, thus introducing difficulties in attempting to define ligands involved in the initial stages of sperm-IPVL interaction.

In this work studies were carried out to further investigate the nature of the carbohydrates involved in sperm-IPVL interaction in chickens. To circumvent the problems of pre-incubation of spermatozoa, IPVL sheets were pre-treated with lectins and glycosidases before assessing the level of sperm-IPVL interaction *in vitro* on the modified layer. However, as the assay relies on spermatozoa both binding to the layer and undergoing acrosomal exocytosis, it was not possible to determine at which stage of the interaction inhibition occurred. Preliminary

studies were also carried out into the role of isolated glycans on the induction of the AR in birds.

4.6.1 Inhibition of IPVL Hydrolysis by Specific Sugar Residues and Specific Lectin Binding

Lectin pre-treatment of the IPVL showed that the layer was able to bind a range of lectins. Of the lectins tested, both FITC-WGA and FITC-S-WGA bound intensely to the IPVL, displaying an even, intense fluorescence over the whole layer (see Table 2; Figure 37). WGA has affinity for GlcNAc and NeuNAc residues (see appendix D for lectin affinities) and the affinity is greatly increased for chitin oligosaccharides (Goldstein and Poretz, 1986), including the chitobiose core of N-linked oligosaccharides (Yamamoto *et al.*, 1981). In contrast S-WGA is modified to yield a lectin which no longer reacts with NeuNAc but retains its other carbohydrate binding properties (Monsigny *et al.*, 1980). Both forms of the lectin completely inhibited sperm hydrolysis of the IPVL, even at a low concentration (see 3.8.1.2) and pre-incubation of WGA with the disaccharide *N,N'*-Diacetylchitobiose, which is a 20 x more potent inhibitor of WGA binding than D-GlcNAc (Goldstein and Poretz, 1986), reversed the inhibitory effect of WGA on sperm hydrolysis of the layer (see 3.8.2).

Lectins from UEA II and STA also recognise D-GlcNAc residues but only in the form of β(1,4)-linked D-GlcNAc oligomers (see appendix D for

lectin specificities). These lectins do not inhibit sperm-IPVL hydrolysis as effectively as WGA, indicating a role for terminal D-GlcNAc residues in sperm-IPVL interaction in chickens. Furthermore, the fluorescence intensity obtained using the FITC-labelled forms of the lectin was less than with WGA, and STA barely showed any staining at all.

Although labelling of the IPVL with FITC-Con A, FITC-PSA and FITC-PNA resulted in a reasonable level of fluorescence they only produced a small reduction (< 30 %) in the number of IPVL-holes at both concentrations used. The first two lectins have affinity for the monosaccharides ∝-D-man and ∝-D-Glu, and PNA for the disaccharide Gal(β-1-3)GalNAc and to a lesser extent D-Gal (see appendix D for lectin specificities). These results indicate that although these sugars are present on the IPVL, they do not play a major role in sperm-IPVL interaction. The fucose binding lectin LTA, displayed only limited fluorescence, indicating a lack of available ∝-L-fuc residues on the IPVL and inhibited hydrolysis of the IPVL by approximately 34 %.

Although lectins are a useful tool for determining specific sugar residues present on the IPVL involved in sperm-IPVL interaction, results should be interpreted cautiously. There are several reasons for the inhibition of sperm hydrolysis of the IPVL including: i) steric inhibition of sperm-IPVL interaction due to binding of the lectin to sites close to the sperm binding protein ii): rearrangement of membrane proteins that prevent sperm-egg interaction iii): cross linking the IPVL fibres which may

render the IPVL resistant to the action of sperm associated proteases. However, although a small decrease in sperm hydrolysis of the IPVL was seen with all lectins used, only WGA and S-WGA completely inhibited hydrolysis of the IPVL. This inhibition was retained at a low concentration suggesting that these lectins may act to block specific D-GlcNAc sites on the IPVL, thus preventing sperm binding.

To further investigate the nature of specific carbohydrates involved in binding of spermatozoa to the IPVL, several saccharides were added to the *in vitro* sperm-IPVL assay (see Section 2.6.3.3 for protocol) to determine their ability to inhibit sperm-IPVL interaction. Of the monosaccharides tested, only D-GlcNAc significantly reduced the number of successful sperm-IPVL interaction events (see Section 3.8.3). The inhibitory effect of this sugar was unlikely to be due to a reduction in sperm motility as at the concentration used, the percentage sperm motility was found to increase from 69% to 78%. (Lena Holm, unpublished results). The reason for this is unknown, but may be due to a stimulatory effect on sperm metabolism by the sugar. Furthermore, inhibition of sperm-IPVL interaction was not caused by induction of the AR by D-GlcNAc as incubation of spermatozoa with the sugar did not result in a significant increase in exposed acrosomes as determined by FITC-PNA labelling (see 3.8.4). In combination with the lectin binding data, these results provide good evidence that terminal D-GlcNAc is important in mediating sperm-egg interaction in chickens.

No significant inhibition of IPVL hydrolysis was seen following the addition of the monosaccharides D-glu, D,gal, D-fuc, L-fuc or D-man to the sperm-IPVL assay, which corresponds with the results obtained with lectin inhibition of IPVL hydrolysis (see Section 3.8.1.2 for results). However, a small, but significant, increase in sperm hydrolytic activity towards the IPVL was found following the addition of D-man to the assay although the reason for this is not known.

The addition of the polysaccharide fucoidin to the sperm-IPVL assay resulted in a small, but significant decrease in the percentage of IPVL-holes. Fucoidin has been shown to be a potent inhibitor of sperm-ZP recognition in several mammalian species including guinea pig, human, hamster (Huang *et al.*, 1982) and rat (Shalgi, *et al.*, 1986). However, in this work the addition of fucoidin to the sperm-IPVL assay reduced sperm hydrolysis of the layer by less than 25%, indicating that it if this molecule is important, it may only have a small role to play in sperm-IPVL interaction in chickens. Neither the fucose binding lectins (UEA II and LTA) nor L-fucose inhibited sperm-IPVL interaction indicating that a terminal fucose residue is not involved in sperm binding.

The results presented here indicate that D-GlcNAc residues located on the IPVL are important in mediating sperm-IPVL interaction in chickens. D-GlcNAc has also been implicated as an important sugar residue in sperm-egg interaction in mammals. WGA has been shown to block fertilization of hamster eggs and this effect is prevented in the

presence of D-GlcNAc (Oikawa, and Yanagimachi, 1973). In mice, several studies have indicated that spermatozoa initially bind to the ZP by an interaction between β-D-GlcNAc, present on the non-reducing terminus of an O-linked oligosaccharide on ZP3 and GlalTase on the sperm surface (Miller *et al.*, 1992; Shur, 1993; Gong *et al.*, 1995).

In mammals various other sugar residues have been proposed to have a role in sperm-zona interaction. For example, in rats GalNAc, GlcNAc, man, ∝-methyl mannosidase, L-fuc and fucoidin were shown to be effective inhibitors of sperm-egg binding (Shalgi *et al.*, 1986), whereas in mice ∝-gal (Bleil and Wassarman, 1988; Johnson *et al.*, 1998), ∝-man (Cornwall *et al.*, 1991), D-GlcNAc (Miller *et al.*, 1992) and fucose (Johnson *et al.*, 1998) residues have all be implicated. It may be that the sperm binding site of ZP3 consists of several sugars. and the variability in the sugars present may account for the species-specificity of sperm-egg interaction. However, in chickens none of the other saccharides or lectins used significantly inhibited hydrolysis of the layer by spermatozoa.

4.6.2 Role of Oligosaccharides in Sperm-IPVL Interaction

In mammals all ZP glycoproteins studied so far are highly glycosylated and possess both O- and N- linked oligosaccharides. (see e.g. Wassarman, 1988; Noguchi and Nakano, 1993; Hokke *et al.*, 1994; Nagdas *et al.*, 1994). In the mouse, ZP3 has been shown to consist of six

potential N-glycosylation sites, 3-4 of which are occupied by complex type N-glycans and an indeterminate number of O-liked sugars attached to more than 70 potential sites (Salzmann *et al.*, 1983; Wassarman *et al.*, 1984). The putative chicken sperm binding protein has been identified as a 34 kDa protein in laid eggs (Waclawek *et al.*, 1998), or a 42 kDa protein in follicular ova (Takeuchi *et al.*, 1999), by its high degree of homology to mammalian ZP3s, and was found to contain only a single N-glycosylated site (Waclawek *et al.*, 1998). However, no functional studies were carried out to determine if this protein was in fact the sperm binding protein on the IPVL.

Pre-treatment of the IPVL with endoglycosidases at equivalent activities (see 2.8.3 for method) demonstrated that removal of N-linked, but not O-linked glycans reduced the ability of spermatozoa to bind to and form points of hydrolysis in the layer. The orcinol sugar assay was used to ensure that sugars were being removed from the IPVL (see Section 2.10 for method) and showed that both O- and N-linked glycans were present in the IPVL. This assay causes hydrolysis of all glycosidic linkages in glycans and the subsequent dehydration of the released monosaccharides. These monosaccharides react with the orcinol to give coloured products. However different sugars produce different colour intensities which makes accurate determination of the total sugar concentration difficult (see White and Kennedy, 1996). Incubation of released O-linked glycans with orcinol produced an orange sugar solution,

whereas the released N-linked glycans produced a paler yellow colour indicating that different monosaccharides were present.

Following removal of N-linked glycans from the IPVL sheets, no difference was seen in the apparent molecular weights of the 78 kDa or the 39 kDa band following SDS-PAGE under reducing conditions. However, removal of O-linked glycans caused a small increase in the molecular weight of the 39 kDa band to approximately 38.5 kDa. (see Section 3.8.5.1). These results are in contrast to those obtained by Waclawek *et al.* (1998), who found a reduction in the putative chZPC band, in both follicular and laid PVL following removal of N-linked glycans with Endo F and an almost undetectable change in the molecular weight of this protein following treatment with O-glycanase. However, several reasons are possible for the different results obtained. Waclawek *et al.*, (1998) removed the glycans from isolated chZP3 and used non-reducing SDS-PAGE to determine the apparent molecular weights of the deglycosylated protein. Furthermore, O-linked glycans were removed in the presence of sialidase only and this may have prevented effective cleavage of the glycan chains.

In mice it is generally accepted that spermatozoa recognise and bind to a specific class of O-linked oligosaccharides found on purified ZP3. Florman and Wassarman (1985) used mild alkaline hydrolysis (β-elimination) to selectively remove O-linked oligosaccharides from mouse ZP3 and demonstrated that sperm binding activity was lost. In contrast,

they found that ZP3 treated with Endo F, to selectively remove N-linked glycans, retained sperm binding activity indicating a role for O- but not N-linked glycans in sperm-egg interaction in mice. However, in contrast to this, preliminary results by Yamagata (1985) demonstrated that removal of N-linked glycans with almond glycopeptidase greatly reduced the ability of spermatozoa to bind to intact ova. It has been suggested that a possible reason for this discrepancy is the substrate specificity of the enzymes used to remove N-linked glycans from purified ZP3 (Nagdas *et al.*, 1994). This group suggests that N-glycanse, the enzyme used by Yamagata (1985), cleaves the β-aspartyl-glucosamine bond between asparagine and the inner most N-acetylglucosamine of all classes of N-linked glycoproteins. However, Endo F, the enzyme used by Florman and Wassarman (1985) may not have been as effective at removing all classes of N-linked glycans, suggesting a role for the remaining N-linked glycans in sperm-egg interaction in mouse.

A similar situation exists in pigs where contradictory evidence has been presented as to whether N-linked glycans (Yonezawa *et al.*, 1995; 1997) or O-linked glycans (Yurewicz *et al.*, 1991) have sperm binding activity. Whatever the situation, it is clear that carbohydrates are involved in mediating sperm-egg interaction in mammals and it may be that N-linked glycans are important in some species while in others O-linked glycans, or both, are used.

4.6.3 Assessment of the Ability of Isolated Glycans to Induce the Acrosome Reaction In Chicken Spermatozoa

Although oligosaccharide chains present on mouse ZP3 have been shown to be important in mediating sperm-zona binding, small ZP3 glycopeptides and ZP3 O-linked oligosaccharides bind spermatozoa but fail to induce the AR (Florman *et al.*, 1984; Florman and Wassarman, 1985; Layton and Saling, 1989). Wassarman and Litscher (1995) suggest that induction of the AR requires multivalent interactions between ZP3 oligosaccharides and egg binding proteins on the spermatozoa. This may lead to a redistribution of the sperm binding protein, thus allowing fusion of that portion of the spermatozoa plasma membrane with the OAM and exocytosis of the acrosomal contents.

In chickens, preliminary results suggest that, unlike the mammalian system, isolated IPVL glycans, released by sequential β-elimination and acid hydrolysis (see Section 3.8.6 for results), are capable of inducing the AR. However, as the sugar fraction used contained mixed glycans it could not be ascertained whether N- or O-linked glycans were involved in induction of the AR. These results must be termed preliminary as removal of the glycans were the result of a single experiment, although the AR assay was carried out on the isolated glycans using three separate samples of spermatozoa. However, further work has confirmed the ability of IPVL liberated glycans to induce the AR in chicken spermatozoa and has shown that N-linked, but not O-linked glycans, have AR inducing

activity (Jackson, 1998). This suggests that the protein backbone of the sperm biding protein on the IPVL is not required for induction of the AR, either indirectly in the presentation of the oligosaccharides to the spermatozoa, or directly as a binding site for spermatozoa.

A similar situation is found in sea urchins, where N-linked glycans released from the egg jelly coat are capable of inducing the AR in spermatozoa (Keller and Vacquier, 1994). Interestingly, liberated O-linked glycans are able to inhibit sperm-egg binding but do not induce the AR (Dhume and Lennarz, 1995).

These results suggest that the IPVL sperm-binding protein contains N-linked glycan(s) with core GlcNAc moieties, possibly with terminal GlcNAc residues. These glycans appear to be involved in mediating sperm-IPVL and interaction and induction of the AR.

CHAPTER 5 SUMMARY

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Currently we have considerable understanding of the mechanisms involved in sperm-egg interaction in mammals, many other vertebrates and invertebrate species, but very little is know about this process in birds. The development of suitable *in vitro* assays for studying the interaction between spermatozoa and ova are valuable for investigating the mechanisms involved in sperm binding and induction of the AR and also for estimating the quality and therefore, potentially, the fertilizing ability of semen (e.g. see Gamzu *et al.*, 1992). In birds, equivalent assays for studying the interaction between spermatozoa and the IPVL have not been well characterized and, as they involve sacrifice of the hen, are limited by the amount of material available, as only one ovulated and a limited amount of follicles can be obtained per bird.

The suitability of IPVL separated from laid chicken eggs was investigated as a readily available, and plentiful, source of material for studying sperm-egg interaction *in vitro*, and for assessing the quality of fresh and liquid-stored spermatozoa. The IPVL was separated from the OPVL following incubation in 0.01 mol l^{-1} HCL and the peptide profiles of the two layers were found to be distinct following separation by SDS-PAGE. Peptide profiles of IPVL obtained from ovulated, follicular and laid eggs were similar to each other, with a 78 kDa band present in IPVL from all three sources. However, in IPVL from laid eggs the 39 kDa band showed greater mobility at 35 kDa. The reason for the increased mobility

of the 39 kDa IPVL protein in laid eggs is not known, but these changes are unlikely to provide a chemical block to penetration of the spermatozoa, as the separated inner layer still retains sperm-binding and activating functions. Incubation of fragments of IPVL from ovulated, follicular and laid eggs were found to have a similar response to hydrolysis by spermatozoa in a 5 min assay at 40°C, showing that neither the acid hydrolysis nor the laying down of the outer layer affects the ability of spermatozoa to bind and hydrolyse the IPVL.

Characterization of the *in vitro* sperm-IPVL assay showed that initiation of hydrolysis was found to have occurred within 2.5 min, with only the size of the holes, not the frequency, increasing with time. The optimum time for the assay was found to be 5 min after which time the holes began to merge. The frequency of points of hydrolysis in IPVL from laid eggs was found to increase linearly with increasing concentration of spermatozoa in the medium. Variation in the frequency of the points of hydrolysis was found to be small in IPVL taken from the same laid egg, for a given concentration of spermatozoa, but greater variability was found between fragments of IPVL from different eggs. It was possible to obtain approximately 20 replicate pieces of IPVL from a single egg, which could be used as an 'experimental unit', providing a good method for studying and manipulating sperm-IPVL interactions *in vitro.*

The development of reliable methods of evaluating sperm quality is important in the poultry industry, both for selecting breeding males and for

assessing the fertilizing ability of semen that has been stored. Therefore, the *in vitro* sperm-IPVL assay was applied as a quantitative test of sperm 'quality' for both fresh and liquid stored semen. It is known that individual males differ in sperm quality and therefore, fertilizing ability. The sperm-IPVL assay was able to highlight differences in sperm quality in semen from different male birds and these results correlated with the results of other sperm quality assays. The results of the sperm-IPVL assay also correlated logarithmically with fertility and linearly with the number of points of hydrolysis produced in the IPVL, at areas away from the GD region, in inseminated hens. Although several *in vitro* assays of sperm quality exist which correlate with fertility in fresh semen, the major advantage of the *in vitro* sperm-IPVL assay, is that it is relatively unsophisticated, requiring only a simple low-power microscope.

Current *in vitro* sperm quality assays have been shown to be poor predictors of fertilizing ability for stored semen. The main problem is that these tests only assess a single factor, such as motility or metabolic activity, and tend to overestimate the fertilizing ability of semen that has been stored. After storage under aerobic and anaerobic conditions at 5°C, the sperm-IPVL assay was able to detect more damaged spermatozoa than the dye exclusion test or the tetrazolium dye reduction assay, indicating that subtle changes occurred during storage that could not be detected by the standard sperm quality assays. Although aeration of chicken spermatozoa during storage at low temperatures has been shown to improve maintenance of fertilizing ability, only the sperm-IPVL assay

detected any difference in sperm quality between the two methods of storage, with anaerobically stored spermatozoa showing a greater impairment in the ability to hydrolyse the layer. The *in vitro* sperm-IPVL assay may therefore, highlight the true nature of the effect of storage of chicken semen at 5°C.

It is generally accepted that turkey semen is more difficult to store *in vitro* than fowl semen and spermatozoa from these birds are known to have an absolute requirement for oxygen for ATP production. The dye exclusion test and the tetrazolium dye reduction assay were virtually unable to distinguish between samples stored under aerobic or anaerobic conditions. However, the ability of aerobically stored spermatozoa to hydrolyse the layer in the *in vitro* sperm-IPVL assay was drastically reduced to approximately 30%, and this function was virtually lost in samples stored under anaerobic conditions. It appears that most of the sperm quality assays are limited in the parameters they measure. The sperm-IPVL assay however, requires that spermatozoa retain a range of functions required of them *in vivo* to fertilize the ovum, including motility, binding to the IPVL, induction of the AR and hydrolysis of the layer. The *in vitro* sperm-IPVL assay therefore, appears to be highly discriminatory against compromised spermatozoa. The fact that it is 'low tech' should allow for its application in selecting and screening individual male birds based on their potential fertilizing ability, and also for the development of suitable semen storage techniques.

The *in vitro* sperm-IPVL assay was also used to assess the role of the IPVL in the species-specific interaction of gametes, by assessing the ability of chicken spermatozoa to hydrolyse the IPVL from different avian species. Chicken spermatozoa were found to hydrolyse the IPVL from all species assessed, but interacted more readily with IPVL from the order *Galliformes*. However, variations were found between and within the different subfamilies indicating that the relationship between the extent of sperm-IPVL interaction and relatedness of species may not be directly related to species divergence within the order *Galliformes*. Cross-reactivity was also seen following incubation of chicken spermatozoa with zebra finch and duck IPVL, which belong to different orders, but to a much lesser degree, suggesting a lack of recognition molecules required for sperm-IPVL interaction, related to species divergence. No interaction was found following incubation of mammalian spermatozoa with chicken IPVL.

In chickens, as in several other avian species, spermatozoa have been shown to preferentially hydrolyse the IPVL overlying the GD in the intact ovum. The reasons for this differential hydrolysis are currently not known and were therefore investigated further. Following co-incubation of intact freshly-ovulated ova with spermatozoa *in vitro*, IPVL-holes were found to be concentrated approximately 7.5 times over the GD region, compared with other areas of the ovum. However, when isolated IPVL fragments from non-GD areas were exposed to the same incubation conditions as the intact ova, the number of holes increased by approximately 8 fold to numbers comparable to those at the GD region in

the intact ovum. Furthermore, when pieces of IPVL with the yolk still attached were incubated in the *in vitro* sperm-IPVL assay, the number of holes were as few as in the intact ovum. This suggests that rather than being attracted to the GD region, spermatozoa are in fact inhibited from hydrolysing the IPVL at areas away from this region by the presence of yolk material.

Development of a suitable routine method of determining the acrosomal status of chicken spermatozoa is essential for studying the mechanisms involved. Of the methods tested, only FITC-PNA reliably detected acrosome reacted chicken spermatozoa following incubation with preparations of IPVL. Electron micrographs revealed that PNA bound to the exposed acrosomes of acrosome reacted, but not acrosome intact spermatozoa. FITC-PNA also bound to the exposed acrosomes of turkey and quail spermatozoa following incubation with homologous IPVL. At 40°C, the avian body temperature, the AR is induced by preparations of IPVL in the presence of Ca^{2+}. However, at 30°C no extracellular Ca^{2+} was required and the IPVL was the sole activator of the AR. A23187 and digitonin did not expose the acrosome in chicken spermatozoa.

Carbohydrates were shown to play an important role in mediating sperm-egg interaction in birds. Following the addition of a range of monosaccharides to the sperm-IPVL assay, only D-GlcNAc significantly inhibited hydrolysis of the layer. The involvement of this sugar was further demonstrated by the effect of preincubation of the IPVL with various

lectins. FITC-WGA showed the most intense fluorescence and was the only lectin to completely inhibit sperm hydrolysis. Removal of N-linked, but not O-linked glycans inhibited sperm-IPVL interaction. Furthermore preliminary results suggest that isolated mixed glycans are capable of inducing the AR in chicken spermatozoa. Therefore these results suggest that chicken sperm-IPVL interaction involves recognition of N-linked glycans with terminal GlcNAc saccharides. Furthermore, unlike the mammalian system, the glycan portion of the IPVL is capable of inducing the AR.

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APPENDICES

APPENDIX A COMPOSITION OF CHICKEN AND TURKEY SEMEN DILUENTS

A.1 Chicken Semen Diluent, pH 7.1

Sodium glutamate.H_2O	1.52 g
Tri-potassium citrate.H_2O	0.128 g
Magnesium acetate.$4H_2O$	0.08 g
Glucose	0.60 g
BES (N,N-Bis(2 hydroxyethyl)-2 aminoethane sulphonic acid	3.05 g

dissolved in distilled water and the pH adjusted to 7.1 with 1 mol NaOH l^{-1} before being made up to a final volume of 100 ml. Stock solutions were divided into 10 ml aliquots and stored at -20°C.

A.2 Turkey Semen Diluent, pH 7.1

Sodium glutamate.H_2O	1.1 g
Sodium acetate (anhydrous)	0.146 g
Tri-potassium citrate.H_2O	0.128 g
Magnesium acetate.$4H_2O$	0.08 g
Glucose	0.36 g
BES	3.05g

dissolved in distilled water and the pH adjusted to 7.1 with 1 mol NaOH l^{-1} before being made up to a final volume of 100 ml. Stock solutions were divided into 10 ml aliquots and stored at -20°C.

A.3 '92G' Diluent

Sodium glutamate.H_2O	1.92 g
Magnesium acetate.$4H_2O$	0.08 g
Tri-potassium citrate.H_2O	0.128 g
Sodium acetate (anhydrous)	0.51 g
Glucose	0.60 g

dissolved in distilled water before being made up to a final volume of 100 ml. Stock solutions were divided into 10 ml aliquots and stored at -20°C.

APPENDIX B **MOUSE FERTILIZATION MEDIUM**

B.1 Stock A

Sodium chloride	5.534g
Potassium chloride	0.356g
potassium dihydrogen orthophosphate	0.162g
Magnesium sulphate.$7H_20$	0.294g
Sodium lactate	2.608g
D-glucose	1.0g
Penicillin	0.6g
Streptomycin sulphate	0.05g

dissolved in 100 ml of double distilled water, filter sterilised using a millipore filter and stored at -20°C.

B.2 Stock B

Sodium hydrogen carbonate	0.42g
Phenol red	0.002g

dissolved in 20 ml of double distilled water and stored at -20°C.

B.3 Stock C

Sodium pyruvate	0.036g

dissolved in 10 ml of double distilled water and stored at -20°C.

B.4 Stock D

Calcium chloride.$2H_20$	0.2g

dissolved in 10 ml of double distilled water and stored at -20°C.

B.5 Mouse Fertilization Medium

Stock A	2.5 ml
Stock B	2.5 ml
Stock C	0.25 ml
Stock D	0.25 ml
BSA	100 mg
Double distilled water	19.5 ml

The medium was filter sterilised before use using a millipore filter and equilibriated for ≥ 10 h in 5% CO_2 before use.

APPENDIX C **REAGENTS AND ANTIDOTE FOR TETRAZOLIUM DYE REDUCTION ASSAY**

C.1 Tetrazolium Dye Reduction Assay Reagents

NaCl-TES	1280 µl
100 mM glucose	100 µl
100 mM potassium cyanide (KCN)*	20 µl
1 mM PMS	50 µl
4 mM INT	100 µl

Stock solutions of NaCl-TES and KCN were stored at 5°C and glucose and INT were stored at -20°C. PMS was prepared fresh as required and stored in light shielded container.

C.2 Potassium Cyanide Antidote

KCN is a lethal poison, therefore an antidote must be prepared fresh each time KCN is used. This consists of:-

15.8% ferrous sulphate ($FeSO_4$) with 1% citric acid in 50 ml of water

6% sodium carbonate (Na_2CO_3) in 50 ml of water.

Mix reagents together immediately before drinking

APPENDIX D **TABLE OF LECTIN SPECIFICITIES**

LECTIN	**CARBOHYDRATE AFFINITY ***
Arachis hypogea (PNA)	Gal(β-1-3)GalNAc > Gal
Canavalia ensiformis (Con A)	∝-Man > ∝-β-Glu> ∝-GlcNAc
Pisum sativum (PSA)	∝-man > ∝-Glu = ∝GlcNAc
Solanum tuberosum (STA)	GlcNAc(β1,4GlcNAc)$_{1-4}$
Tetragonolobus purpureas (LTA)	α-L-Fuc > L-Fucα1,2Galβ1,4GalNAc > L-Fucα1,2Galβ1,3GalNAc
Triticum vulgaris (WGA)	GlcNAc(β1,4GlcNAc)$_{1-2}$ > βGlcNAc > NeuNAc
Ulex europaeus II (UEA II)	L-Fucα1,2Galβ1,4GlcNAc > GlcNAc(β1-4GlcNAc)$_{1-3}$

* Saccharide-binding specificities of Lectins as quoted by Goldstein and Poretz (1986)

APPENDIX E. **REAGENTS FOR SDS-PAGE**

E.1 Boiling Mix

stacking gel buffer	1.0 ml
25% SDS	0.8 ml
β-mercaptoethanol	0.5 ml
glycerol	1.0 ml
bromophenol blue (0.2% w/v)	0.05 ml

E.2 Resolving gel buffer

Tris(hydroxymethy)methylamine (Tris)	45.38 g
SDS	1.00 g

dissolved in distilled water and the pH adjusted to 8.9 with conc. HCL before being made up to a final volume of 250 ml. Stock solutions were stored at -20°C.

E.3 Stacking gel buffer

Tris	14.73 g
SDS	1.00 g

dissolved in distilled water and the pH adjusted to 6.7 with conc. HCL before being made up to a final volume of 250 ml. Stock solutions were stored at -20°C.

E.4 5-15% Gradient Resolving Gel

Acrylamide used to prepare gradient gels was Phigel 1 (19:1 acrylamide: N,N-methylenebisacrylamide 40% stock solution) (Fisons Scientific Equipment, Bishop Meadow Road, Longborough).

5-15% Gradient Resolving Gel Mixture

	5%	15%
40% acrylamide (Phigel 1),	4.4 ml	12.0 ml
resolving gel buffer	6.0 ml	6.0 ml
distilled water	14.0 ml	2.4 ml
Glycerol	-	3.6 ml

Stock solutions were stored at 5°C until required.

6 ml of each of the above stocks were used to pour two mini-gels, polymerisation was activated by the addition of the following to each solution:-

10% (w/v) ammonium persulphate	50 µl
TEMED	2.5 µl

E.5 5% Stacking gel mixture

30% Acrylamide	4 ml
Stacking gel buffer	6 ml
distilled water	14 ml

Stock solutions were stored at 5°C until required.

6 ml of the above stock was used to pour two stacking gels, polymerisation was activated by the addition of the following:-

10% (w/v) ammonium persulphate	50 µl
TEMED	2.5 µl

E.6 Electrode Buffer

tris	15.8 g
glycine	10.0 g
SDS	2.5 g

dissolved in 2.5 ml of distilled water

E.7 Components of 'Electran' Molecular Weight Markers

	M.W (kDa).
Cytochrome C	12.3
Myoglobin	17.2
Carbonic anhydrase	30.0
Ovalbumin	42.7
Albumin	66.25
Ovotransferrin	76-78.0

E.8 Silver Staining

Solution	Reagents	Time
Fix	50% methanol 12% acetic acid 0.5 ml 37% formaldehyde / litre	1 h (min)
Wash A	50% ethanol	3 x 20 min
Pretreat	0.2 g sodium thiosulphate / litre	1 min
Rinse	dH_2O	3 x 20 sec
Impregnate	2g silver nitrate / litre 0.7 ml 37% formaldehyde / litre 4 mg sodium thiosulphate / litre	20 min
Rinse	dH_2O	3 x 20 sec
Develop	60 g sodium carbonate / litre 0.5 ml 37% formaldehyde / litre 4 mg sodium thiosulphate / litre	As long as required
Rinse	dH_2O	3 x 20 sec
Stop	50% methanol 12% acetic acid	10 min
Wash B	50% methanol	20 min
Store	50% methanol	3-4 weeks

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